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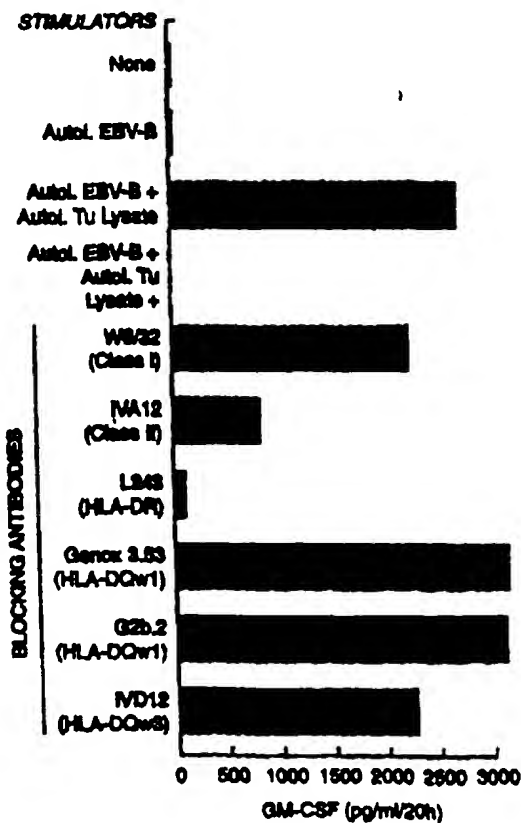## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(54) Title: MHC-CLASS II RESTRICTED MELANOMA ANTIGENS AND THEIR USE IN THERAPEUTIC METHODS

(57) Abstract

The present invention provides MHC Class II restricted melanoma antigens recognized by CD4+ T cells. This invention further provides prophylactic and therapeutic applications for the Class II restricted melanoma antigens. In particular, this invention provides tyrosinase Class II restricted melanoma antigens, as well as tyrosinase immunogenic peptides which have been modified to enhance their immunogenicity. These antigens can serve as immunogens or vaccines to prevent or treat melanoma. In addition a method for isolating Class II restricted melanoma antigens or identifying new Class II restricted melanoma antigens is provided.

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TITLE OF THE INVENTION

MHC-CLASS II RESTRICTED MELANOMA ANTIGENS AND THEIR USE IN THERAPEUTIC METHODS

FIELD OF THE INVENTION

This invention is in the field of prevention and treatment of human cancers. More specifically, this invention relates to MHC Class II restricted melanoma antigens recognized by helper T Cells and to the preventative, and therapeutic applications which employ these antigens. This invention also relates to methods for determining Class II restricted melanoma antigens.

BACKGROUND OF THE INVENTION

Melanomas are aggressive, frequently metastatic tumors derived from either melanocytes or melanocyte related nevus cells ("Cellular and Molecular Immunology" (1991) (eds) Abbas A.K., Lechtman, A.H., Pober, J.S.; W.B. Saunders Company, Philadelphia: pages 340-341). Melanomas make up approximately three percent of all skin cancers and the worldwide increase in melanoma is unsurpassed by any other neoplasm with the exception of lung cancer in women ("Cellular and Molecular Immunology" (1991) (eds) Abbas, A.K., Lechtiman, A.H., Pober, J.S.; W.B. Saunders Company Philadelphia pages: 340-342; Kirkwood and Agarwala (1993) Principles and Practice of Oncology 7:1-16). Even when melanoma is apparently localized to the skin, up to 30% of the patients will develop systemic metastasis and the majority of these will die (Kirkwood and Agarwala (1993) Principles and Practice of Oncology 7:1-16). Classic modalities of treating melanoma include surgery, radiation and chemotherapy. In the past decade immunotherapy and gene therapy have emerged as new and promising methods for treating melanoma (Biologic Therapy of Cancer, 2nd ed. (1995) Devita, V.T., Hellman, S, and Rosenberg, S.A., eds; J.B. Lippincott Co., Philadelphia).

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Shared melanoma-associated antigens (Ag) expressed among a variety of melanoma patients can be recognized by cytotoxic CD8^{+} T lymphocytes derived from melanoma patients. In short-term lysis assays, cytotoxic T lymphocytes (CTL) grown from in vitro sensitized peripheral blood lymphocytes (PBL) or lymph node lymphocytes, or from lymphocytes infiltrating metastatic melanoma lesions, have been shown to recognize autologous and MHC class I compatible allogeneic melanomas but not HLA-matched nonmelanoma tumors, lymphoblasts, or cultured fibroblasts (Darrow, D.L., Slingluff, C.L., & Siegler, H.F. (1989.) *J. Immunol.* **142**, 3329-3335; Hom, S.S., et al. (1991) *J. Immunother.* **10**, 153-164). Similar recognition patterns have been observed by measuring cytokine secretion from tumor infiltrating lymphocytes (TIL) cocultivated with autologous or HLA-matched allogeneic tumor stimulators (Hom, S.S., et al. (1993) *J. Immunother.* **13**, 18-30). Recently, melanoma-specific HLA-A2 restricted CTL clones have been shown to recognize cultured normal melanocytes as well as their malignant counterparts, suggesting that shared melanoma antigens can be lineage specific (Anichini, A., et al. (1993) *J. Exp. Med.* **177**, 989-998). To date, several class I-restricted melanoma-associated antigens have been molecularly defined (Van Der Bruggen, P., et al. (1991) *Science* **254**, 1643-1647; Brichard, V., et al. (1993) *J. Exp. Med.* **178**, 489-495; Kawakami, Y., et al. (1994) *Proc. Natl. Acad. Sci. USA.* **91**, 3515-3519; Bakker, A.B.H., et al. (1994) *J. Exp. Med.* **179**, 1005-1009; Kawakami, Y., et al. (1994) *Proc. Natl. Acad. Sci. USA.* 91,6458-6462; Gaugler B., et al. (1994) *J. Exp. Med.* **179**, 921-930). These antigens and derivative class I-restricted peptides 8 to 10 amino acids in length are currently being developed as clinical vaccines to stimulate CD8^{+} T cell responses against melanoma.

While animal models of malignant and viral diseases have shown the importance of CD8^{+} T cells in the

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effector phase of the immune response, the CD4⁺ helper arm has been shown to mediate critical priming and effector functions as well. T cell receptors on CD4⁺ T cells recognize a complex consisting of an antigenic peptide in conjunction with an MHC Class II molecule. Unlike
5   peptides binding to MHC class I molecules, which are restricted in length from 8-10 amino acids, the antigenic peptides that bind Class II range from about 10 to about 34 amino acids in length and even entire proteins. (Chicz, R.M. et al (1993) *J. Exp. Med.* 178, 27-47; Sette,
10   A. et al (1989) *J. Immunol* 143, 1265-1267.) This is due to the structure of the peptide binding groove in MHC class II molecules, which is open at both ends and allows for overhang of longer peptides outside of the critical binding core. In contrast, the peptide binding groove in
15   MHC class I molecules is closed at both ends, strictly limiting the length of possible binding peptides. (Brown, J.H. et al (1993) *Nature* 364, 33-39).

        Strong and long lasting immunity depends in part on CD4⁺ helper T cell functions. Therefore the
20   identification of Class II-restricted melanoma antigens will broaden the immunotherapeutic approaches to treating and/or prophylaxing against melanoma.

SUMMARY OF THE INVENTION

        This invention relates, in general, to MHC Class
25   II restricted melanoma antigens recognized by CD4⁺T-lymphocytes and the nucleic acid sequences encoding these antigens. This invention also provides therapeutic uses for the nucleic acid sequences, proteins or peptides described herein. In addition, this invention provides a
30   method for identifying additional Class II restricted melanoma antigens.

        It is a general object of the present invention to provide proteins, polypeptides or peptides which encode for Class II restricted melanoma antigens.

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It is another object of this invention to provide a recombinant molecule comprising a vector and all or part of the nucleic acid sequence encoding for a Class II restricted melanoma antigen.

It is another object of this invention to produce recombinant proteins or peptides encoded by all or part of the nucleic acid sequence encoding for a Class II restricted melanoma antigen.

It is a further object of this invention to provide methods for prophylactic or therapeutic uses for the Class II restricted melanoma antigens.

It is also an object of this invention to provide melanoma vaccines comprising all or part of the Class II restricted melanoma antigens.

It is a further object of this invention to provide immunogenic peptides demonstrated to be Class II restricted melanoma antigens for use in vaccines.

It is a particular object of this invention to provide tyrosinase peptides which are Class II restricted melanoma antigens.

In addition, it is another object of this invention to provide multivalent vaccines comprising at least one Class II restricted melanoma antigen and at least one other immunogenic molecule capable of eliciting an immune response in a mammal to melanoma antigens.

It is another object of this invention to provide a method for preventing or treating melanoma utilizing Class II restricted melanoma antigens in gene therapy protocols.

It is a further object of this invention to provide peptides derived from a tyrosinase protein sequence for use in vaccines.

It is yet another object of this invention to provide a method of prophylactic or therapeutic immunization for melanoma using the vaccines described herein.

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It is a further object of this invention to provide a method of identifying Class II restricted melanoma antigens that would constitute potential targets for immunotherapy.

DESCRIPTION OF THE FIGURES

Figure 1 shows that the response of CD4$^+$ tumor infiltrating lymphocytes (TIL) from patient number 1088 to autologous melanoma cells is HLA-DR restricted. TIL (1 x 10^6/ml) were cultured in the presence of autologous Epstein Barr Virus (EBV)-B cells (1 x 10^6/ml) alone, or B cells pulsed with a lysate of autologous cultured melanoma cells (7 x 10^5 cell equivalents/ml). Secretion of GM-CSF (granulocyte/macrophage-colony-stimulating factor) following autologous tumor stimulation was significantly inhibited by the monoclonal antibody (mAb) L243 (anti-HLA-DR) and IVA12 (anti-HLA-DR,-DP,-DQ).

Figure 2 shows CD4$^+$ TIL 1088 recognize lysates of autologous and allogeneic cultured melanoma lines presented by autologous EBV-B cells, indicating a commonly expressed melanoma antigen. TIL cultured for 58 days in the presence of IL-2 were incubated for 20 hours (h) with tumor-pulsed 1088-EBV. GM-CSF secretion was measured by ELISA. All cells were at 1 x 10^6/ml.

Figure 3 shows CD4$^+$ TIL 1088 react with melanoma cells and normal melanocytes, but not with tumors of other histologies. TIL secreted significant amounts of GM-CSF when stimulated with autologous EBV-B cells pulsed with lysates of the autologous 1088-mel or normal melanocytes FM 902 and FM 907, but not pulsed with lysates of colon cancers, breast cancers, or Ewing's sarcomas. All cells were 10^6 per ml. Ca, cancer; Sa; sarcoma

Figure 4 shows that CD4$^+$ TIL 1088 recognize two different tyrosinase peptides. HLA-DRB1*0401 was identified as the presenting molecule for both Ty 56-70 and Ty 448-462. CD4$^+$TIL 1088 recognition was assessed by GM-CSF secretion. Peptides HA 307-319 and MT(65) 3-13

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were used as inhibitors for B1*0401 and B1*0301, respectively.

Figure 5 shows the MHC Class II restriction of tyrosinase peptide recognition by CD4$^+$ TIL 1088 cells. The antigen presenting cells were pulsed with autologous melanoma lysate or 100 μM peptide overnight then washed prior to assay, all cells at 1 x 10^6/ml. HLA-DR-B1*0401 was identified as the presenting MHC molecule for Ty 56-70 and Ty 448-462.

Figure 6 shows in three separate experiments, CD4$^+$T cells from patient 1088 showed specific recognition of the Ty 56-70 peptide. Mutated and truncated peptides were used to identify the primary anchor (binding) residues within Ty 56-70. Recognition was measured as GM-CSF secretion by T cells cocultured for 24 hours with peptide-pulsed autologous EBV-transformed B cells.

Figure 7 shows the tyrosinase (Ty) peptides Ty 56-70 and Ty 448-462 with the P1 and P6 anchor positions boxed. The number designation of the peptide (e.g. Ty 56-70) indicates the amino acids spanned by the peptide, with 1 being the methionine encoded by the initiation codon.

Figure 8 shows CD4$^+$1088 TIL recognize modified tyrosinase 56-70 (Ty 56-70) peptides better than the nonmutated or non-modified peptide. (Background TIL + EBV = 118 pg/ml, subtracted.)

Figure 9 shows in two experiments, Ty 448-462 was specifically recognized by CD4$^+$T cells from patient 1088. Mutated and truncated peptides were used to identify Y451 as the primary P1 binding residue within Ty 448-462.

Figure 10 shows CD4$^+$ TIL 1088 recognize truncated tyrosinase 448-462 peptides, Ty 449-462 and Ty 450-462, better than Ty 448-462.(Background TIL + EBV = 196 pg/ml, subtracted).

Figure 11 shows the P6 anchor position for Ty 448-462 is D456. This was presumed, based on identifying

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Y451 as the P1 anchor. D is not an optimal residue in this position, and a valine substitution led to markedly enhanced CD4$^+$ T cell recognition. When two favorable modifications of Ty 448-462 were combined in a single modified peptide (Ty 450-462, D456 → V), recognition was enhanced even more (see Figure 12).

Figure 12 shows CD4$^+$ TIL 1088 recognize modified Tyrosinase 448-462 peptides better than the non-modified or nonmutated peptide (Background TIL + EBV = 99 pg/ml, subtracted).

DETAILED DESCRIPTION OF THE INVENTION

Major Histocompatibility Complex (MHC) is a generic designation meant to encompass the histocompatibility antigen systems described in different species, including the human leucocyte antigens (HLA).

The term melanoma includes, but is not limited to, melanomas, metastatic melanomas, melanomas derived from either melanocytes or melanocyte related nevus cells, melanocarcinomas, melanoepitheliomas, melanosarcomas, melanoma in situ, superficial spreading melanoma, nodular melanoma, lentigo maligna melanoma, acral lentiginous melanoma, invasive melanoma or familial atypical mole and melanoma (FAM-M) syndrome. Such melanomas in mammals may be caused by, chromosomal abnormalities, degenerative growth and developmental disorders, mitogenic agents, ultraviolet radiation (UV), viral infections, inappropriate tissue expression of a gene, alterations in expression of a gene, and presentation on a cell, or carcinogenic agents. The aforementioned melanomas can be diagnosed, assessed or treated by methods described in the present application.

Immunogenic peptide includes, but is not limited to, an antigenic peptide capable of causing or stimulating a cellular or humoral immune response. Such peptides may also be reactive with antibodies.

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This invention provides MHC-Class II restricted melanoma antigens. Such antigens may be the complete protein encoded by a gene, or portions thereof or polypeptides or peptides derived from a protein sequence. Such antigens may be expressed in normal or disease tissues. By way of example, Class II restricted melanoma antigens may be derived from the tyrosinase amino acid sequences. Examples of immunogenic tyrosinase sequences that may be used include, but are not limited to, GenBank accession numbers J03581, U01873, Y00819, and M27160, (Kwon, et al., (1987) *PNAS* 84:7473-7477; Brichard, V. et al., (1993) *J. Exp. Med.* 178:489-495; Bouchard, B. et al. (1989) *J. Exp. Med.* 169:2029-2042; and Shibihara, S. et al., (1988) *J. Exp. Med.* 156:403-414; all herein incorporated by reference). The Class II restricted melanoma antigen may comprise the entire tyrosinase sequence or portions thereof. Examples of immunogenic tyrosinase peptides recognized by CD4$^+$ T cells include, but are not limited to, QNILLSNAPLGPQFP (Ty 56-70) (SEQ ID NO: 1), NILLSNAPLGPQFP (Ty 57-70) (SEQ ID NO: 2), DYSYLQDSDPDSFQD (Ty 448-462) (SEQ ID NO: 6), YSYLQDSDPDSFQD (Ty 449-462) (SEQ ID NO: 13), and SYLQDSDPDSFQD (Ty 450-462) (SEQ ID NO: 14) (Peptides are presented in single letter code). Also intended to be encompassed by this invention are proteins or polypeptides comprising these immunogenic peptide sequences. Persons of ordinary skill in the art will recognize that these peptides could be shortened to a minimal MHC Class II binding core of 9 or 10 amino acids by truncating the amino and/or carboxy termini of these peptides or one could lengthen these peptides by adding flanking sequences at either the carboxy or amino terminus of the peptides or at both termini of the peptide. By way of example, such a peptide may range in size from about 9 amino acids to about 34 amino acids.

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This invention further includes analogs of these immunogenic peptides derived from the tyrosinase amino acid sequence. The term analog includes any peptide which displays the functional aspects of these immunogenic peptides. The term analog also includes conservative substitution or chemical derivative of the peptides as described above. These peptides may be synthetically or recombinantly produced by conventional methodology.

The term "analog" includes any polypeptide having an amino acid residue sequence substantially identical to the sequences described herein in which one or more residues have been conservatively substituted with a functionally similar residue and which displays the functional aspects of the peptides as described herein. Examples of conservative substitutions include the substitution of one non-polar (hydrophobic) residue such as isoleucine, valine, leucine or methionine for another, the substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, between glycine and serine, the substitution of one basic residue such as lysine, arginine or histidine for another, or the substitution of one acidic residue, such as aspartic acid or glutamic acid for another.

The phrase "conservative substitution" also includes the use of a chemically derivatized residue in place of a non-derivatized residue. "Chemical derivative" refers to a subject polypeptide having one or more residues chemically derivatized by reaction of a functional side group. Examples of such derivatized molecules include for example, those molecules in which free amino groups have been derivatized to form amine hydrochlorides, p-toluene sulfonyl groups, carbobenzoxy groups, t-butyloxycarbonyl groups, chloroacetyl groups or formyl groups. Free carboxyl groups may be derivatized to form salts, methyl and ethyl esters or other types of

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esters or hydrazides. Free hydroxyl groups may be
derivatized to form O-acyl or O-alkyl derivatives. The
imidazole nitrogen of histidine may be derivatized to form
N-im-benzylhistidine. Also included as chemical
derivatives are those proteins or peptides which contain
one or more naturally-occurring amino acid derivatives of
the twenty standard amino acids. For examples: 4-
hydroxyproline may be substituted for proline; 5-
hydroxylysine may be substituted for lysine; 3-
methylhistidine may be substituted for histidine;
homoserine may be substituted for serine; and ornithine
may be substituted for lysine. Proteins, polypeptides or
proteins of the present invention also include any
polypeptide having one or more additions and/or deletions
or residues relative to the sequence of a polypeptide or
peptide whose sequence are described herein, so long as
the requisite activity is maintained.

In yet another embodiment of this invention,
Class II restricted peptides derived from a tyrosinase
sequence are modified to increase immunogenicity by
enhancing the binding of the peptide to the MHC Class II
molecule with which the peptide is associated when
presented to $CD4^+$ T cells, or by enhancing binding of the
peptide to the T cell receptor of the $CD4^+$ T cells. By
way of example, modifications may include the
substitution, deletion or addition, of one or more amino
acids within the peptide sequence, or insertion of amino
acids within the given peptide sequence or derivitization
of existing amino acids within the given peptide sequence
or mutation of the amino acids within the given peptide
sequence. Examples of modified QNILLSNAPLGPQFP (Ty 56-70)
(SEQ ID NO: 1) peptides include, but are not limited to,
QNILLSNAPVGPQFP (L65→V) (SEQ ID NO: 3), QNILLSNVPVGPQFP
(A63→V and L65→V) (SEQ ID NO: 4), and QNILLSNVPLGPQFP
(A63→V) (SEQ ID NO: 5) (See Figure 6). Examples of
modified DYSYLQDSDPDSFQD (Ty 448-462) (SEQ ID NO: 6)

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peptides include, but are not limited to DYSYLQDSDPDSSQD
(F460→S) (SEQ ID NO: 7), DQSYLQDSDPDSFQD (Y449→Q) (SEQ ID
NO: 8), DFSYLQDSDPDSFQD (Y449→F) (SEQ ID NO: 9),
DYSFLQDSDPDSFQD (Y451→F) (SEQ ID NO: 10), DYSYLQDSVPDSFQD
(D456→V) (SEQ ID NO: 11), and SYLQDSVPDSFQD (Ty450-462,
D456→V) (SEQ ID NO: 12) (see Figures 9 and 11).

Preferably the modifications are performed
within the Class II core binding of the tyrosinase
peptides. In a preferred modification at least one amino
acid is substituted or replaced in the given binding core
of the immunogenic peptide sequence. Any amino acid
composing the given binding core of the immunogenic
peptide sequence may be modified in accordance with this
invention. Any amino acid may be used to substitute or
replace a given amino acid within the binding core of the
immunogenic peptide sequence. Modification may occur at
any amino acid position within the binding core of an
immunogenic tyrosinase peptide. Modified peptides is
intended to include any modified peptide exhibiting
enhanced binding with the MHC Class II molecule with which
it is associated when presented to the CD4$^+$ T cell. Also
intended to be encompassed by this invention are proteins
or polypeptides comprising or including these peptide
sequences. By way of example such proteins or
polypeptides may have additional sequences such as
flanking sequences either at the carboxy or amino terminus
of the peptide or both.

By way of example, the Class II restricted
tyrosinase antigens may be recognized by CD4$^+$T cells in
the context of HLA-DR, in particular HLA-DRB1*0401. The
core binding sequence of a Class II restricted antigen is
about 9 amino acids in length. Preferably for enhanced
binding of the peptide to HLA-DRB1*0401 the first position
in the core amino acid sequence is an aromatic or
aliphatic hydrophobic amino acid. The sixth position may
be any hydrophobic amino acid such as, but not limited to,

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leucine, isoleucine, valine, methionine, or a hydroxyl amino acid, such as serine or threonine (Sette, A. et al (1993) *J. Immunol.* 151, 3163-3170; Rammensee, H.G. et al (1995) *Immunogenetics* 41:178-228, both herein incorporated by reference).

The fourth, seventh and ninth positions of the 9 amino acid binding core sequence of the immunogenic peptide may also be substituted or replaced. Examples of amino acids that may be used at the fourth position of the peptide include, but are not limited to, any hydrophobic amino acid or aspartic or glutamic acid. The seventh position may be any polar, charged or aliphatic amino acid. Examples of amino acids that may be used include but are not limited to aspartic acid, alanine, serine, valine, histidine, proline, asparagine, methionine, threonine, leucine and isoleucine. The ninth position of the peptide may be any polar or aliphatic amino acid. Examples of such amino acids include but are not limited to alanine, serine, glutamine, glycine, leucine, valine, and threonine.

Examples of Class II restricted tyrosinase peptides whose core sequence may be modified in accordance with the present embodiment include, but is not limited to QNILLSNAPLGPQFP (Ty 56-70), NILLSNAPLGPQFP (Ty 57-70), DYSYLQDSDPDSFQD (Ty 448-462), YSYLQDSDPDSFQD (Ty 449-462), and SYLQDSDPDSFQD (Ty 450-462). Examples of modified tyrosinase peptides whose core binding sequence may further be modified include, but is not limited to, QNILLSNAPVGPQFP (Ty56-70, L65→V), QNILLSNVPVGPQFP (Ty 56-70, A63→V and L65→V), QNILLSNVPLGPQFP (Ty 56-70, A63→V), DYSYLQDSDPDSSQD (Ty 448-462, F460→S), DQSYLQDSDPDSFQD (Ty 448-462, Y449-Q), DFSYLQDSDPDSFQD (Ty 448-462, Y449→F), DYSFLQDSDPDSFQD (Ty 448-462, Y451→F), DYSYLQDSVPDSFQD (Ty 448-462, D456→V), and SYLQDSVPDSFQD (Ty450-462, D456→V).

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By way of example modified Class II-restricted tyrosinase peptides derived from the tyrosinase sequence may have a binding core sequence according to the formula $X_1LLX_2NX_3X_4LX_5$ (SEQ ID NO: 15), or $X_1LQX_2SX_3X_4DX_5$ (SEQ ID NO: 16), wherein:

5   X_1 may be any hydrophobic amino acid, either aromatic or aliphatic. Examples of amino acids that may be used include, but are not limited to, leucine, isoleucine, methionine, valine, tryptophan, phenylalanine, or tyrosine. The X_1 position corresponds to Ty 58 in

10   peptide Ty56-70, or Ty 451 in peptide Ty 448-462.

X_2 may be any hydrophobic amino acid, or aspartic acid or glutamic acid. Examples of amino acids that may be used include, but are not limited to, phenylalanine, tryptophan, leucine, isoleucine, valine,

15   alanine, aspartic acid or glutamic acid.

X_3 may be any hydrophobic amino acid, or hydroxyl amino acids. Examples of amino acids that may be used include, but are not limited to, leucine, isoleucine, methionine, valine, serine or threonine.

20   X_4 may be any polar, charged or aliphatic amino acid. Examples of amino acids that may be used include, but are not limited to, aspartic acid, alanine, serine, valine, histidine, proline, asparagine, methionine, threonine, leucine, and isoleucine.

25   X_5 may be any polar, or aliphatic amino acid. By way of example amino acids that may be used on this position include, but are not limited to, alanine, serine, glutamine, glycine, leucine, valine, and threonine.

This invention further includes analogs of these

30   modified peptides derived from the tyrosinase sequence. The term analog is intended to include any peptide which displays the functional aspects of these modified peptides as described above. These modified peptides may be synthetically or recombinantly produced by conventional

35   methods. Also intended to be encompassed by this

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invention are proteins or polypeptides including these peptide sequences. By way of example such proteins or polypeptides may have additional flanking sequences at either the carboxy or amino terminus of the peptide or at both termini or may be truncated to a minimal 9 amino acid
5   MHC Class II binding core.

This invention also relates to nucleic acid encoding the Class II restricted immunogenic tyrosinase peptides or modified peptides of this invention. It is, however, understood by one skilled in the art that due to
10   the degeneracy of the genetic code variations in nucleic acid sequences will still result in a DNA sequence capable of encoding antigens described herein. Such DNA sequences are therefore functionally equivalent to the sequences intended to be encompassed by the invention. Allelic
15   variations in a given species of the nucleic acid sequence encompassed by this invention are also intended to be encompassed by the present invention. By way of example nucleic acid sequences encoding the tyrosinase or modified tyrosinase peptides QNILLSNAPLGPQFP (Ty 56-70),
20   NILLSNAPLGPQFP (Ty 57-70), DYSYLQDSDPDSFQD (Ty 448-462), YSYLQDSDPDSFQD (Ty 449-462), SYLQDSDPDSFQD (Ty 450-462), QNILLSNAPVGPQFP (Ty 56-70, L65→V), QNILLSNVPVGPQFP (Ty 56-70, A63→V and L65→V), QNILLSNVPLGPQFP (Ty 56-70, A63→V) DYSYLQDSDPDSSQD (Ty 448-462, F460→S), DQSYLQDSDPDSFQD (Ty
25   448-462, Y449→Q), DFSYLQDSDPDSFQD (Ty 448-462, Y449→F), DYSFLQDSDPDSFQD (Ty 448-462, Y451→F), DYSYLQDSVPDSFQD (Ty-448-462, D456→V), and SYLQDSVPDSFQD (Ty450-462, D456→V) or analogs thereof are intended to be encompassed by this invention.

30   This invention also provides a recombinant DNA molecule comprising all or part of the nucleic acid sequence encoding a Class II melanoma antigen and a vector. Expression vectors suitable for use in the present invention comprise at least one expression control
35   element operationally linked to the nucleic acid sequence.

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The expression control elements are inserted in the vector
to control and regulate the expression of the nucleic acid
sequence. Examples of expression control elements
include, but are not limited to, lac system, operator and
promoter regions of phage lambda, yeast promoters and
5   promoters derived from polyoma, adenovirus, retrovirus or
SV40. Additional preferred or required operational
elements include, but are not limited to, leader sequence,
termination codons, polyadenylation signals and any other
sequences necessary or preferred for the appropriate
10   transcription and subsequent translation of the nucleic
acid sequence in the host system. It will be understood
by one skilled in the art that the correct combination of
required or preferred expression control elements will
depend on the host system chosen. It will further be
15   understood that the expression vector should contain
additional elements necessary for the transfer and
subsequent replication of the expression vector containing
the nucleic acid sequence in the host system. Examples of
such elements include, but are not limited to, origins of
20   replication and selectable markers. It will further be
understood by one skilled in the art that such vectors are
easily constructed using conventional methods (Ausubel et
al., (1987) in "Current Protocols in Molecular Biology",
John Wiley and Sons, New York, New York) or are
25   commercially available.

        Another aspect of this invention relates to a
host organism into which a recombinant expression vector
containing all or part of the nucleic acid sequence
encoding for a Class II melanoma antigens has been
30   inserted. The host cells transformed with the nucleic
acid sequences encompassed by this invention include
eukaryotes, such as animal, plant, insect and yeast cells
and prokaryotes, such as E. coli. The means by which the
vector carrying the gene may be introduced into the cell
35   include, but are not limited to, microinjection,

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electroporation, transduction, or transfection using DEAE-
dextran, lipofection, calcium phosphate or other
procedures known to one skilled in the art (Sambrook et
al. (1989) in "Molecular Cloning. A Laboratory Manual",
Cold Spring Harbor Press, Plainview, New York).

5   In a preferred embodiment, eukaryotic expression
vectors that function in eukaryotic cells are used.
Examples of such vectors include, but are not limited to,
retroviral vectors, vaccinia virus vectors, adenovirus
vectors, herpes virus vector, fowl pox virus vector,

10   plasmids, such as pCDNA3 (Invitrogen, San Diego, CA) or
the baculovirus transfer vectors. Preferred eukaryotic
cell lines include, but are not limited to, COS cells, CHO
cells, HeLa cells, NIH/3T3 cells, 293 cells (ATCC#
CRL1573), T2 cells, dendritic cells, monocytes or Epstein-

15   Barr Virus transformed B cells. In a preferred embodiment
the recombinant protein expression vector is introduced
into mammalian cells, such as NIH/3T3, COS-7, CHO, 293
cells (ATCC #CRL 1573), T2 cells, dendritic cells, or
monocytes to ensure proper processing and modification of

20   the protein.

           The recombinant protein expressed by the host
cells can be obtained as a crude lysate or can be purified
by standard protein purification procedures known in the
art which may include differential precipitation,

25   molecular sieve chromatography, ion-exchange
chromatography, isoelectric focusing, gel electrophoresis,
affinity, and immunoaffinity chromatography and the like.
(Ausubel et. al., (1987) in "Current Protocols in
Molecular Biology" John Wiley and Sons, New York, New

30   York). In the case of immunoaffinity chromatography, the
recombinant protein may be purified by passage through a
column containing a resin which has bound thereto
antibodies specific for the tyrosinase protein (Ausubel
et. al., (1987) in "Current Protocols in Molecular

35   Biology" John Wiley and Sons, New York, New York).

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This invention further includes an antibody or antibodies reactive with the Class II restricted melanoma antigens described. The antibodies may be monoclonal and polyclonal and are made by conventional methods known to those skilled in the art. In addition, the protein or
5   nucleic acid sequences of the Class II restricted melanoma antigens described herein, may be used diagnostically to screen for the presence, absence or alteration in expression of these antigens using immunoassays or nucleic acid probes.

10   The Class II restricted melanoma antigens of this invention, or analogs thereof may be used as a vaccine either prophylactically or therapeutically. When provided prophylactically the vaccine is provided in advance of any evidence of melanoma. The prophylactic
15   administration of the Class II restricted melanoma antigen vaccine should serve to prevent or attenuate melanoma in a mammal. In a preferred embodiment mammals, preferably human, at high risk for melanoma are prophylactically treated with the vaccines of this invention. Examples of
20   such mammals include, but are not limited to, humans with a family history of melanoma, humans with a history of atypical moles, humans with a history of FAM-M syndrome or humans afflicted with melanoma previously resected and therefore at risk for reoccurrence. When provided
25   therapeutically, the vaccine is provided to enhance the patient's own immune response to the tumor antigen present on the melanoma or metastatic melanoma. The vaccine, which acts as an immunogen, may be a cell, cell lysate from cells transfected with a recombinant expression vector,
30   cell lysates from cells transfected with a recombinant expression vector encoding for the Class II restricted melanoma antigen, or a culture supernatant containing the expressed protein. Alternatively, the immunogen is a partially or substantially purified recombinant protein,
35   peptide or analog thereof encoding for a Class II

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restricted melanoma antigen. The proteins or peptides may
be conjugated with lipoprotein or administered in
liposomal form or with adjuvant using conventional
methodologies. Examples of Class II restricted tyrosinase
peptides or modified tyrosinase peptides that may be used
5   include, but are not limited to, QNILLSNAPLGPQFP (Ty 56-
70), NILLSNAPLGPQFP (Ty 57-70), DYSYLQDSDPDSFQD (Ty 448-
462), YSYLQDSDPDSFQD (Ty 449-462), SYLQDSDPDSFQD (Ty 450-
462), QNILLSNAPVGPQFP (Ty 56-70, L65→V), QNILLSNVPVGPQFP
(Ty 56-70, A63→V and L65→V), QNILLSNVPLGPQFP (Ty 56-70,
10   A63→V), DYSYLQDSDPDSSQD (Ty 448-462, F460→S),
DQSYLQDSDPDSFQD (Ty 448-462, Y449→Q), DFSYLQDSDPDSFQD (Ty
448-462, Y449→F), DYSFLQDSDPDSFQD (Ty 448-462, Y451→F),
DYSYLQDSVPDSFQD (Ty 448-462, D456→V), and SYLQDSVPDSFQD
(Ty450-462, D456→V) or analogs thereof. The tyrosinase
15   protein or tyrosinase peptides having the modified binding
core sequences described herein may also be used.

        While it is possible for the immunogen to be
administered in a pure or substantially pure form, it is
preferable to present it as a pharmaceutical composition,
20   formulation or preparation.

        The formulations of the present invention, both
for veterinary and for human use, comprise an immunogen as
described above, together with one or more pharma-
ceutically acceptable carriers and, optionally, other
25   therapeutic ingredients. The carrier(s) must be
"acceptable" in the sense of being compatible with the
other ingredients of the formulation and not deleterious
to the recipient thereof. The formulations may
conveniently be presented in unit dosage form and may be
30   prepared by any method well-known in the pharmaceutical
art.

        All methods include the step of bringing into
association the active ingredient with the carrier which
constitutes one or more accessory ingredients. In
35   general, the formulations are prepared by uniformly and

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intimately bringing into association the active ingredient
with liquid carriers or finely divided solid carriers or
both, and then, if necessary, shaping the product into the
desired formulation.

Formulations suitable for intravenous,
5   intramuscular, subcutaneous, or intraperitoneal
administration conveniently comprise sterile aqueous
solutions of the active ingredient with solutions which
are preferably isotonic with the blood of the recipient.
Such formulations may be conveniently prepared by
10   dissolving solid active ingredient in water containing
physiologically compatible substances such as sodium
chloride (e.g. 0.1-2.0M), glycine, and the like, and
having a buffered pH compatible with physiological
conditions to produce an aqueous solution, and rendering
15   said solution sterile. These may be present in unit or
multi-dose containers, for example, sealed ampoules or
vials.

The formulations of the present invention may
incorporate a stabilizer. Illustrative stabilizers are
20   polyethylene glycol, proteins, saccharides, amino acids,
inorganic acids, and organic acids which may be used
either on their own or as admixtures. These stabilizers
are preferably incorporated in an amount of about 0.11
about 10,000 parts by weight per part by weight of
25   immunogen. If two or more stabilizers are to be used,
their total amount is preferably within the range
specified above. These stabilizers are used in aqueous
solutions at the appropriate concentration and pH. The
specific osmotic pressure of such aqueous solutions is
30   generally in the range of about 0.1 to about 3.0 osmoles,
preferably in the range of about 0.8 to about 1.2. The pH
of the aqueous solution is adjusted to be within the range
of about 5.0 to about 9.0, preferably within the range of
6-8. In formulating the immunogen of the present
35   invention, anti-adsorption agent may be used.

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Additional pharmaceutical methods may be employed to control the duration of action. Controlled release preparations may be achieved through the use of polymer to complex or absorb the proteins or their derivatives. The controlled delivery may be exercised by selecting appropriate macromolecules (for example polyester, polyamino acids, polyvinyl, pyrrolidone, ethylenevinylacetate, methylcellulose, carboxymethylcellulose, or protamine sulfate) and the concentration of macromolecules as well as the methods of incorporation in order to control release. Another possible method to control the duration of action by controlled-release preparations is to incorporate the tyrosinase protein, peptides and analogs thereof into particles of a polymeric material such as polyesters, polyamino acids, hydrogels, poly(lactic acid) or ethylene vinylacetate copolymers. Alternatively, instead of incorporating these agents into polymeric particles, it is possible to entrap these materials in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxy-methylcellulose or gelatin-microcapsules and poly(methylmethacylate) microcapsules, respectively, or in colloidal drug delivery systems, for example, liposomes, albumin microspheres, microemulsions, nanoparticles, and nanocapsules or in macroemulsions.

When oral preparations are desired, the compositions may be combined with typical carriers, such as lactose, sucrose, starch, talc magnesium stearate, crystalline cellulose, methyl cellulose, carboxymethyl cellulose, glycerin, sodium alginate or gum arabic among others.

The proteins of the present invention may be supplied in the form of a kit, alone, or in the form of a pharmaceutical composition as described above.

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Vaccination can be conducted by conventional
methods. For example, the immunogen can be used in a
suitable diluent such as saline or water, or complete or
incomplete adjuvants. Further, the immunogen may or may
not be bound to a carrier to make the protein immunogenic
or enhance the protein's immunogenecity. Examples of such
carrier molecules include but are not limited to bovine
serum albumin (BSA), keyhole limpet hemocyanin (KLH),
tetanus toxoid, and the like. The immunogen also may be
coupled with lipoproteins or administered in liposomal
form or with adjuvants. The immunogen can be administered
by any route appropriate for antibody production such as
intravenous, intraperitoneal, intramuscular, subcutaneous,
and the like. The immunogen may be administered once or
at periodic intervals until a significant titer of $CD4^+$ or
$CD8^+$ T cell or antibodies directed against the Class II
restricted melanoma antigen is obtained. The presence of
cells may be assessed by measuring cytokine secretion in
response to antigen-presenting cells pulsed with the
immunogen. The antibody may be detected in the serum
using conventional immunoassays.

The administration of the vaccine or immunogen
of the present invention may be for either a prophylactic
or therapeutic purpose. When provided prophylactically,
the immunogen is provided in advance of any evidence or in
advance of any symptom due to melanoma, or in patients
rendered free of disease by conventional therapies but at
significant risk for recurrence. The prophylactic admini-
stration of the immunogen serves to prevent or attenuate
melanoma in a mammal. When provided therapeutically, the
immunogen is provided at (or after) the onset of the
disease or at the onset of any symptom of the disease.
The therapeutic administration of the immunogen serves to
attenuate the disease.

By way of example, a vaccine prepared using
recombinant expression vectors may be used. To provide a

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vaccine to an individual a genetic sequence which encodes
for all or part of the Class II restricted melanoma
antigen is inserted into an expression vector, as
described above, and introduced into the mammal to be
immunized. Examples of vectors that may be used in the
aforementioned vaccines include, but are not limited to,
defective retroviral vectors, adenoviral vectors, vaccinia
viral vectors, fowl pox viral vectors, or other viral
vectors (Mulligan, R.C., (1993) Science 260:926-932). The
viral vectors carrying the nucleic sequence can be
introduced into a mammal either prior to any evidence of
melanoma or to mediate regression of the disease in a
mammal afflicted with melanoma. Examples of methods for
administering the viral vector into the mammals include,
but are not limited to, exposure of cells to the virus ex
vivo, or injection of the retrovirus or a producer cell
line of the virus into the affected tissue or intravenous
administration of the virus. Alternatively the viral
vector carrying all or part of the tyrosinase nucleic acid
sequence encoding the Class II restricted melanoma antigen
may be administered locally by direct injection into the
melanoma lesion or topical application in a
pharmaceutically acceptable carrier. Examples of nucleic
acid sequences that may be used include, but are not
limited to, nucleic acid sequence encoding the Class II
tyrosinase restricted peptides or modified peptides
QNILLSNAPLGPQFP (Ty 56-70), NILLSNAPLGPQFP (Ty 57-70),
DYSYLQDSDPDSFQD (Ty 448-462), YSYLQDSDPDSFQD (Ty 449-462),
SYLQDSDPDSFQD (Ty 450-462), QNILLSNAPVGPQFP (Ty 56-70,
L65→V), QNILLSNVPVGPQFP (Ty 56-70, A63→V and L65→V),
QNILLSNVPLGPQFP (Ty 56-70, A63→V), DYSYLQDSDPDSSQD (Ty
448-462, F460→S), DQSYLQDSDPDSFQD (Ty 448-462, Y449-Q),
DFSYLQDSDPDSFQD (Ty 448-462, Y449→F), DYSFLQDSDPDSFQD (Ty
448-462, Y451→F), DYSYLQDSVPDSFQD (Ty 448-462, D456→V),
and SYLQDSVPDSFQD (Ty450-462, D456-V) or analogs thereof.
In addition, nucleic acid sequences encoding tyrosinase

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peptides comprising the modified core binding sequences
described herein may also be incorporated into recombinant
vectors. The quantity of viral vector, carrying the
nucleic acid sequence encoding for the Class II restricted
melanoma antigen, to be administered is based on the titer

5   of virus particles. By way of example, a range of the
immunogen to be administered may be about 10^6 to about 10^{11}
virus particles per mammal, preferably a human. After
immunization the efficacy of the vaccine can be assessed
by production of antibodies or immune cells that recognize

10   the antigen, as assessed by specific cytokine production
or by tumor regression. One skilled in the art would know
the conventional methods to assess the aforementioned
parameters. If the mammal to be immunized is already
afflicted with melanoma or metastatic melanoma the vaccine

15   can be administered in conjunction with other therapeutic
treatments. Examples of other therapeutic treatment
includes, but are not limited to, adoptive T cell
immunotherapy, coadministration of cytokines or other
therapeutic drugs for melanoma.

20   Alternatively all or parts thereof of a
substantially or partially purified tyrosinase protein
corresponding to the Class II restricted melanoma antigen
or polypeptides or peptides may be administered as a
vaccine in a pharmaceutically acceptable carrier. By way

25   of example, ranges of protein polypeptides or peptides to
be administered may be 0.001 to about 100 mg per patient,
preferred doses are about 0.01 to about 10mg per patient.
In a preferred embodiment, tyrosinase Class II restricted
peptide melanoma antigens or analogs thereof or modified

30   tyrosinase peptides are administered therapeutically or
prophylactically to a mammal in need of such treatment.
By way of example, doses may be about 0.001 mg to about
100 mg, preferred doses are about 0.01 mg to about 10mg.
The peptide may be synthetically or recombinantly

35   produced. Immunization may be repeated as necessary,

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until a sufficient titer of anti-immunogen antibody or reactive CD4$^+$ or CD8$^+$ T cells has been obtained.

In yet another alternative embodiment a viral vector, such as a retroviral vector, can be introduced into mammalian cells. Examples of mammalian cells into which the retroviral vector can be introduced include, but are not limited to, primary mammalian cultures or continuous mammalian cultures, COS cells, NIH3T3, or 293 cells (ATTC #CRL 1573), B cell, dendritic or monocytic cell cultures. The means by which the vector carrying the gene may be introduced into a cell includes, but is not limited to, microinjection, electroporation, transfection or transfection using DEAE dextran, lipofection, calcium phosphate or other procedures known to one skilled in the art (Sambrook et al. (eds) (1989) in "Molecular Cloning. A Laboratory Manual", Cold Spring Harbor Press, Plainview, New York). The mammalian cells expressing the Class II restricted melanoma antigen can be administered to mammals and serve as a vaccine or immunogen. Examples of how the cells expressing Class II restricted melanoma antigens can be administered include, but is not limited to, subcutaneous, intravenous, intraperitoneal or intralesional. In a preferred embodiment, the nucleic acid sequence corresponding to Class II restricted peptides or modified tyrosinase peptides is inserted into expression vector and introduced into the mammalian cells. By way of example, the peptides that may be used include, but are not limited to, QNILLSNAPLGPQFP (Ty 56-70), NILLSNAPLGPQFP (Ty 57-70), DYSYLQDSDPDSFQD (Ty 448-462), YSYLQDSDPDSFQD (Ty 449-462), SYLQDSDPDSFQD (Ty 450-462), QNILLSNAPVGPQFP (Ty 56-70, L65→V), QNILLSNVPVGPQFP (Ty 56-70, A63→V and L65→V), QNILLSNVPLGPQFP (Ty 56-70, A63→V) DYSYLQDSDPDSSQD (F460→S), DQSYLQDSDPDSFQD (Ty 448-462, Y449-Q), DFSYLQDSDPDSFQD (Ty 448-462, Y449→F), DYSFLQDSDPDSFQD (Ty 448-462, Y451→F), DYSYLQDSVPDSFQD (Ty 448-462, D456→V), and SYLQDSVPDSFQD (Ty450-462, D456-V) or

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analogs thereof. Nucleic acid sequences encoding
tyrosinase peptides having the binding core sequences
provided may also be used. Conventional methods would be
used to evaluate the immune response of the patient to
determine the efficiency of the vaccine.

In yet another embodiment of this invention, the
Class II restricted tyrosinase protein, peptides or
modified peptides or analogs thereof may be exposed to
dendritic cells cultured in vitro. The cultured dendritic
cells provide a means of producing CD4$^+$ T cell dependent
antigens comprised of dendritic cell modified antigen or
dendritic cells pulsed with antigen, in which the protein
antigen is processed and expressed on the antigen
activated dendritic cell. The antigen activated dendritic
cells or processed dendritic cell antigens may be used as
immunogens for vaccines or for the treatment of melanoma.
Alternatively the dendritic cells may present peptide
antigens which have been pulsed on externally. The
dendritic cells should be exposed to antigen for
sufficient time to allow the antigens to bind directly to
their surface MHC Class II molecules, or to be
internalized and presented on the dendritic cells surface.
The resulting dendritic cells or the dendritic cell
processed antigens can than be administered to an
individual in need of therapy. Such methods are described
in Steinman et al. (WO93/208185) and in Banchereau et al.
(EPO Application 0563485A1) which are incorporated herein
by reference. Monocytes, B cells, or Langerhans cells may
be substituted for dendritic cells.

In yet another embodiment of this invention CD4$^-$
T cells isolated from individuals can be exposed to the
Class II restricted melanoma antigen in vitro and then
administered to a patient in need of such treatment in a
therapeutically effective amount. Examples of where
CD4$^+$T-lymphocytes can be isolated, include but are not
limited to, peripheral blood cells lymphocytes (PBL),

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lymph nodes, or tumor infiltrating lymphocytes (TIL).
Such lymphocytes can be isolated from the individual to be
treated or from a donor by methods known in the art and
cultured in vitro (Kawakami, Y. et al. (1989) J. Immunol.
142: 2453-3461). The CD4$^+$T-lymphocytes are cultured by
methods known in the art. The lymphocytes are exposed to
peptide or protein antigen for part or all of the culture
duration, in the presence of antigen presenting cells. In
a preferred embodiment the CD4$^+$lymphocytes are exposed to
the Class II restricted tyrosinase peptides or any
tyrosinase sequence having the core peptide sequences
described herein. By way of example, a concentration of
about 1 to about 200 micrograms(ug)/ml peptides per 10^7
cells for all or part of the duration of lymphocyte
culture may be used. After being sensitized to the
peptide the T-lymphocytes are administered to the mammal
in need of such treatment. Examples of how these
sensitized CD4$^+$ T cells can be administered to the mammal
include but are not limited to, intravenously,
intraperitoneally or intralesionally. Parameters that may
be assessed to determine the efficacy of these sensitized
T-lymphocytes include, but are not limited to, production
of immune cells in the mammal being treated or tumor
regression. Conventional methods are used to assess these
parameters. Such treatment can be given in conjunction
with cytokines or gene modified cells (Rosenberg, S.A. et
al. (1992) Human Gene Therapy, 3: 75-90; Rosenberg, S.A.
et al. (1992) Human Gene Therapy, 3: 57-73). By way of
example the MHC Class II restricted melanoma antigen may
be administered in conjunction with GM-CSF to enhance
uptake by professional antigen presenting cells in vivo.

In yet another alternative embodiment Class II
restricted melanoma antigens may be linked with MHC Class
II molecules and administered either prophylactically or
therapeutically to mammals. By way of example the
tyrosinase peptides or modified peptides described herein

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may be coupled with an MHC Class II molecule. Such
coupling may be covalent, chemical, or genetic. By way of
example, tyrosinase peptides may be genetically linked to
the Class II β chain, HLA-DRB1*0401, by a flexible peptide
linker which allows the peptide to lie in the binding
5   groove for recognition by T cells (Kozono, H et al (1994)
Nature 369, 151-154, herein incorporated by reference).
According to the promiscuous and degenerate nature of
peptide binding to MHC Class II molecules, other DRB1
chains may be used as well (Sinigaglia, F. et al (1995) J.
10   Exp. Med. 181, 449-451). Such single-chain Class II-MHC-
peptide constructs may be used as vaccines, or may be used
to raise reactive CD4$^+$ T cells in vitro.

In yet another alternative embodiment, the
aforementioned compositions can be used to prepare
15   antibodies to the Class II restricted melanoma antigens.
The antibodies can be used directly as anti-melanoma
therapeutic agents or as diagnostic reagents. Further,
the antibodies can be made even more compatible with the
host system by generating "humanized" chimeric antibodies
20   (Morrison J. 1985, Science 229:1202 and Oi, et al.
Biotechniques (1986) 4:214). Such antibodies can be
generated by conventional methods.

In yet another embodiment of this invention,
multivalent vaccines against one or more melanoma antigens
25   are provided. Such multivalent vaccines may comprise at
least one of the Class II restricted melanoma antigens
described herein, preferably the tyrosinase immunogenic
peptides disclosed herein or combinations thereof,
combined with other known melanoma antigens or peptides
30   derived from these antigens. By way of example, MHC Class
II restricted tyrosinase peptides may be combined in a
vaccine with MHC Class I restricted peptides derived from
tyrosinase or other known melanoma associated proteins to
create a multivalent vaccine capable of stimulating helper
35   and cytotoxic immune cells. Examples of known melanoma

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antigens include, but are not limited to, MART-1, gp100, gp75, MAGE-1 and MAGE-3 or immunogeneic peptides derived from these proteins.

In another embodiment, a method is provided for identifying the presence of Class II restricted antigenic proteins or epitopes of proteins, or of identifying new proteins encoding Class II restricted tumor associated antigens, such as, but not limited to, melanoma antigens. By way of example, the genes or nucleic acid sequences encoding Class I restricted melanoma antigens can be screened for the presence of a Class II restricted antigenic portions or epitopes of the protein encoded by these genes.   In this embodiment, the method may comprise the steps of: (a) exposing a candidate antigen to antigen presenting cells (APC) for a period of time sufficient to allow the APC to take up and process the antigen; (b) incubating the APC of step (a) with CD4$^+$T-lymphocytes; and (c) screening for recognition of the APC by the CD4$^+$ T cells (see Example 1).

In step (a) the candidate antigen may be presented to the APC by either stably on transiently expressing the gene for the candidate antigen in a eukaryotic or prokaryotic expression system.   The antigen may then be presented to the APC as crude lysates of the cells expressing the canidate antigen or as purified protein products from the candidate antigen expressing cells.   Alternatively a plurality of peptides based on the candidate protein amino acid sequence or based on a truncated protein sequence derived from experiments with serial truncations of the candidate gene may be exposed or incubated with the antigen presenting cell.   It is preferred that peptides of about 15 to 20 amino acids be used.

Examples of APC that may be used in step (a) include, but are not limited to, antigen presenting cells such as EBV transformed B cell lines (Topalian et al.

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°   (1994) *Int. J. Cancer* 58:69-79), monocytes and dendritic
cells. Examples of how to assess recognition by the CD4^{+}
T cells incubated with the APC in step (c) include, but
are not limited to, [51]CR release cytotoxicity assays
(Cerundolo, V. et al. (1990) *Nature* 345:449-452.),
5   cytokine secretion assays such as γ-IFN, GM-CSF or TNF
secretion. (Schwartzentruber, D. et al., (1991) *J. of
Immunology* 146:3674-3681), or proliferation assays.

        Examples of proteins that may be screened for
Class II restricted melanoma antigens includes, but are
10   not limited to MART-1 (Kawakami, et al. (1994) *Proc. Natl.
Acad. Sci.* 91:3575-3579), p15 (Robbins, P.F. et al (1995)
J. Immunol. 154:5944-5950), MAGE-1 (VanderBruggen, *Science*
254:1643-1647), gp100 (Kawakami, et al. (1994) 91:6458-
6462), gp75 (Wang, R-F et al (1995) *J. Exp. Med.* 181:799-
15   804), and MAGE-3 (Gaugler, et al. (1994), *J. Exp. Med.*
179:921-930); all herein incorporated by reference.)

        Alternatively, this method can be used to clone
and identify new genes having CD4 recognized tumor
antigens. By way of example, a cell expressing an
20   unidentified tumor antigen would be assessed for CD4^{+} T
cell recognition by pulsing lysates of that cell onto
antigen presenting cells (EBV-cells, monocytes, dendritic
cells, etc.), and measuring cytokine secretion by T cells
during coincubation. A DNA library from the tumor or
25   other stimulatory cell would be expressed in a prokaryotic
or eukaryotic host cell and screened according to the
methods outlined above.

        Also intended to be encompassed by this
invention are Class II restricted tumor associated
30   antigens, such as melanoma antigens obtained by these
methods.

        Veterinary uses are also intended to be
encompassed by the compositions and therapeutic
applications described herein.

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All books, articles, and patents referenced herein are incorporated by reference. The following examples illustrate various aspects of the invention and in no way intended to limit the scope thereof.

Example I

Human CD4$^+$ Cells Recognize a Shared
Melanoma Antigen Encoded by the Tyrosinase Gene

MATERIALS AND METHODS

Lymphocyte Cultures and Clones.

TIL were cultured from enzymatically digested single cell suspensions of solid metastatic melanoma lesions as previously described (Topalian, S.L., et al. (1987) *J. Immunol. Methods.* **102**, 127-141), in the presence of recombinant interleukin-2 (rIL-2; 6000 IU/ml (Chiron Corporation, Emeryville, CA)), Lymphocyte cultures were passaged in bulk for 4 to 6 weeks, and then CD4$^+$ and CD8$^+$ TIL subsets were purified by positive selection on tissue culture flasks carrying covalently bound anti-CD4 or anti-CD8 monoclonal antibodies (mAb) (Applied Immune Sciences, Menlo, Park, CA) (Morecki, S., et al. (1990) *J. Biol. Resp. Modif.* **9**, 463-474). Uncloned CD4$^+$ TIL were tested in bioassays after 45-70 days of culture. CD4$^+$ T cell clones were established from a 35-day bulk TIL 1088 culture which was 52% CD4$^+$. CD4$^+$ T cells were selected and then cloned by limiting dilution in microtiter plates, in the presence of 600 IU/ml IL-2, pooled allogeneic PBL from 3 donor's (total 3 x 10^4 cells/well, 3000 rad; 1 rad = 0.01Gy), and autologous Epstein Barr Virus (EBV)-B cells (1088-EBV 1 x 10^4 cells/well, 10,000 rad) pulsed with a freeze/thaw lysate of autologous tumor (1088-mel). Clones were restimulated weekly with allogeneic peripheral blood lymphocytes (PBL), 1088-EBV, and whole 1088-mel cells (1 x 10^3 cells/well, 30,000 rad). Clones used for bioassays were grown from 0.3 or 1 cell/well dilutions, and tested after 58-155 days of culture.

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B Cell Lines.

EBV-transformed B cell lines were established
from the PBL of melanoma patients using standard
techniques (Current Protocols In Immunology, Coligan, J.
E. et al. (eds) Wiley and Sons, N.Y., N.Y. 7.22.1-7.22.3
(1994) herein incorporated by reference). These lines
were maintained as suspension cultures in RPMI + 10% FCS.
Tumors and Normal Melanocytes.

Melanoma cultures were established from fresh or
cryopreserved single cell suspensions of metastatic
lesions and maintained as adherent monolayers in RPMI +
10% FCS, as described (Topalian, S.L., et al. (1989) J.
Immunol. **142**, 3714-3725). Tumor clones were established
by limiting dilution in 96-well flat bottom plates
(Costar).

The normal melanocyte cultures FM 707, FM 708,
FM 902, FM 906 and FM 907, generated from neonatal
foreskin, were a generous gift of Dr. Meenhard Herlyn
(Wistar Institute, Philadelphia, PA; Herlyn, M., et al.
(1985) Cancer Res. **45**, 5670-5676). Cultures were
maintained in Melanocyte Growth Medium (MGM, Clonetics,
San Diego, CA), a basal serum-free medium supplemented
with recombinant basic fibroblast growth factor (1 ng/ml),
insulin (5 ug/ml), hydrocortisone (0.5 ug/ml), PMA (10
ng/ml), bovine pituitary extract, gentamicin (50 ug/ml),
and amphotericin B (50 ng/ml). Cells were removed from
this medium for several days prior to bioassays.

The cultured colon carcinoma CY13 was a gift of
Dr. J. Yannelli (NIH, Bethesda, MD). Colon carcinomas
WiDr, LoVo, SW480; breast carcinomas ZR-75-1 and MCF7; and
Ewing's sarcomas 6647, RD-ES, and TC-71 were all obtained
from the American Type Culture Collection (ATCC,
Rockville, MD) and maintained in RPMI + 10% FCS. All
cultures tested negative for mycoplasma contamination.

Fresh tumor specimens were prepared from
enzymatically dispersed single cell suspensions of solid

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tumors, including melanomas, colon carcinomas, sarcomas, and lymphomas. They were cryopreserved in 90% FCS + 10% DMSO, and were rapidly thawed for immediate use on the day of bioassay.

Antigen Presentation to T Cells by EBV-Transformed B Cells

Optimization of a bioassay for tumor-reactive CD4$^+$ T cells, using EBV-B cells as antigen presenting cells for lysates of whole tumor cells, has been described (Topalian, S.L., et al. (1994) *Int. J. Cancer.* 58:69-79). Assay medium consisted of RPMI + 10% AB serum with IL-2 120 IU/ml. Briefly, washed EBV-B cells were cultured at 7-10 x 10^5 cells/ml. Tumor antigen was added to B cells in the form of cell lysates: concentrated preparations of washed whole tumor cells were subjected to 3 cycles of rapid freezing and thawing, and cell fragments were added to B cells at 7-10 x 10^5 cell equivalents/ml. Tumor-pulsed B cell cultures were maintained at 37°C for 20-24 h, then TIL were added at 4-10 x 10^5 cells/ml. Cultures were established in flat-bottom 96-well plates (220 ul/well), 48-well plates (550 ul/well), or 24-well plates (1100 ul/well) depending on the numbers of cells available. As a positive control, TIL were also cultured in plates coated with anti-CD3 mAb (OKT3; Ortho Pharmaceuticals, Raritan, NJ). Cultures were maintained for an additional 20-24 hours (h), then supernatants were harvested by centrifugation and stored at -80°C until assayed for the presence of cytokines. Secreted cytokines were measured with ELISA kits purchased from R+D Systems (Minneapolis, MN) for granulocyte-macrophage colony stimulating factor (GM-CSF, detectable concentrations 8-500 pg/ml), tumor necrosis factor alpha (TNF-α, 15-1000 pg/ml), IL-4 (31-2000 pg/ml) and IL-6 (3-300 pg/ml). GM-CSF assays were calibrated with international reference standard 88/646 (NCI-FCRDC, Frederick, MD). Interferon gamma (IFN-γ) was measured with an ELISA developed with

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reagents from Biosource International (Camarillo, CA) (20-10,000 pg/ml).

The response of TIL to tumor stimulation was considered to be significant when cytokine secretion in response to tumor-pulsed EBV-B cells exceeded the response to EBV-B cells alone by ≥3.0-fold.

Antibody Blocking Studies.

To inhibit TIL recognition of tumor-pulsed EBV-B cells, stimulator cells were cultured with preservative-free mAb for 30 min at room temperature before adding TIL, and then cultures were maintained for 24 h at 37°C in the continued presence of mAb 23 ug/ml. Antibodies directed against HLA determinants included W6/32 (against HLA-A,B,C; IgG$_{2a}$; Sera-Lab, Sussex, England), IVA12 (HLA-DR, DP, DQ;IgG), L243 (HLA-DR;IgG$_{2a}$), Genox 3.53 and G2b.2 (HLA-DQw1;IgG, and IgG$_{2a}$, respectively), and IVD12 (HLA-DQw3;IgG) (all purified from ATCC hybridoma supernatants).

Transfection of COS-7 Cells.

Genes cloned into the expression vectors pcDNA3 (Invitrogen, San Diego, CA) or pCEV27 (Miki, T., et al. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 5167-5171) were transiently transfected into the monkey kidney COS-7 cell line (a gift of Dr. W. Leonard, NIH) using the DEAE dextran method (Seed, B., & Aruffo, A. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 3365-3369). EBV-B cells were pulsed with lysates of transfected COS-7 cells for recognition by T cells. The tyrosinase gene was isolated from a cDNA library from the cultured melanoma line 1290A-mel, and its identity was confirmed by partial DNA sequencing which gave a sequence identical to that published by Bouchard (Bouchard, B., et al. (1989) *J. Exp. Med.* **169**, 2029-2042). The tyrosinase (1-3) (comprising exons 1 through 3) gene, also isolated from the 1290A-mel library, lacks the fourth and fifth exons and encodes a truncated product. The gene encoding tyrosinase related protein (gp75) was isolated by screening a cDNA library from 501-mel with a probe

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°   constructed based on the published gene sequence
        (Vijayasaradhi, S., et al. (1990) *J. Exp. Med.* **171**, 1375-
        1380).

        <u>RESULTS</u>

                CD4$^+$ T cells were purified by positive selection
5   from a heterogeneous population of bulk cultured TIL
        derived from a metastatic melanoma lesion from patient
        1088. Selected cultures were >95% CD4$^+$. Preliminary
        experiments indicated that these CD4$^+$ T cells secreted
        cytokines specifically when cocultivated with autologous
10   EBV-B cells (1088-EBV) which had been pulsed with lysates
        of autologous melanoma cells (1088-mel). TIL secreted
        large quantities of GM-CSF, and much smaller quantities of
        TNF-α, IL-4, and IFN-γ in response to autologous tumor;
        specific IL-6 secretion was not observed. Thus, GM-CSF
15   secretion was monitored as a measure of T cell recognition
        in subsequent assays. In 11 separate experiments, CD4$^+$
        TIL stimulated with tumor-pulsed EBV-B cells secreted 8-
        to 138-fold more GM-CSF (median 46-fold) than TIL
        stimulated with EBV-B cells in the absence of tumor. As
20   shown in Figure 1, GM-CSF secretion could be abrogated by
        blocking with the anti-HLA-DR mAb L243 (96% inhibition),
        suggesting that TIL reactivity was HLA-DR restricted.
        Significant blocking of cytokine secretion was also
        observed by the anti-class II framework mAb IVA12 (71%
25   inhibition), but not by isotype-matched mAb directed
        against a monomorphic MHC class I determinant or against
        two HLA-DQ determinants (HLA type of patient 1088: HLA-DR
        4, 17; HLA-DQw2, 3; HLA-DRw52, 53). Conversely, in the
        same experiment, cytokine secretion by purified CD8$^+$ TIL
30   1088 in response to whole autologous melanoma cells (MHC
        class I$^+$, class II$^-$) was inhibited by the anti-class I mAb
        W6/32, but not by L243 or IVA12 (data not shown). This
        experiment was repeated once with similar results. CD8$^+$
        TIL 1088 capable of recognizing whole 1088-mel cells
35   failed to react to 1088-EBV cells pulsed with tumor

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lysate. These data indicate that CD4$^+$ TIL 1088 recognize autologous tumor antigen presented by EBV-B cells in a specific, MHC class II-restricted manner.

A variety of allogeneic tumors and normal tissues were screened for the presence of Ag recognized by CD4$^+$ TIL 1088. A representative experiment is shown in Figure 2, in which lysates of the autologous cultured melanoma line as well as all 7 allogeneic melanoma cultures tested were stimulatory. Stimulation indices [SI = GM-CSF secretion by (TIL + EBV-B + cell lysate)/(TIL + EBV-B)] ranged from 4.2 for 938-mel to 104.8 for 553-mel. TIL alone secreted 22 pg/ml GM-CSF, and when cocultivated with unpulsed 1088-EBV secreted 101 pg/ml, as compared to 10,175 pg/ml secreted in the presence of 1088-EBV pulsed with 1088-mel lysate. In separate experiments, a total of 18 fresh or cultured melanomas were screened for recognition, of which 12 were positive (67%), 4 were negative, and 2 equivocal on repeat experiments. Although tumor 624-mel was recognized by TIL 1088 on 5 separate occasions (SI = 8.0 to 24.8), only 4 of 6 tumor clones from 624-mel were recognized. Taken together, these results suggest that the Ag recognized by CD4$^+$ TIL 1088 are broadly but not universally expressed in melanoma lesions; alternatively, recognition may reflect relative degrees of antigen expression and the sensitivity of our detection system. Of note, TIL recognized autologous fresh melanoma cells (SI=19.0 and 13.1 in two experiments) as well as tumor cultured from the same lesion (1088-mel), from passages 6 through 48. Thus, the recognized antigen were present in vivo and were not a function of culture artifact; their expression was conserved through almost one year of continuous in vitro culture.

Although TIL recognized a number of allogeneic melanomas on repeat assays, they consistently failed to react with normal cells of nonmelanocyte lineage derived from the same patients. For instance, CD4$^+$ TIL 1088

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responded to 1088-EBV pulsed with 1088-mel lysate but not to these same EBV-B cells pulsed with 1088-EBV lysate (SI = 35.7 and 1.4, respectively). This experiment was repeated twice with similar results. Also, TIL secreted significant amounts of GM-CSF in response to a lysate of fresh 501B melanoma cells, but not to lysates of EBV-B cells or cultured fibroblasts derived from the same patient (SI = 13.4, 2.2, and 1.5 respectively).

TIL were tested for recognition of a variety of nonmelanoma tumors, both fresh and cultured. As represented in Figure 3, TIL failed to recognize lysates from 14 tumors of various histologic types, including colon carcinomas, breast carcinomas, lymphomas, and sarcomas. TIL also failed to react with two Ewing's sarcomas, which share a neuroectodermal embryonic origin with melanomas and were recognized by melanoma-specific CD8$^+$ T cells in a previous study (Shamamian, P., et al. (1994) *Cancer Immunol. Immunother*. 39:73-83). However, CD4$^+$ TIL 1088 did recognize all four normal melanocyte lines assayed, on repeated occasions. Measured levels of GM-CSF secretion approached those observed in response to 1088-mel. These results suggested that the Ag recognized by CD4$^+$ TIL 1088 might be specific for the melanocytic lineage.

Two other melanoma patients whose CD4$^+$ TILs recognized lysates of autologous melanoma cells presented by autologous or HLA-matched EBV-B cells had previously been identified (Topalian, S. et al., (1994) *Int. J. Cancer* 58:69-79) by our laboratory. These TILs appeared to be MHC class II-restricted and to recognize antigens unique to autologous melanoma cells, since they failed to react with 15 allogeneic melanomas including 1088-mel or with normal cells including cultured melanocytes. These results suggest the existence of multiple class II-restricted melanoma determinants which are differentially

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° and specifically recognized by CD4$^+$ T cells from these three patients.

TIL cultured in bulk under the conditions described have been shown to be oligoclonal, but not monoclonal cell populations (Belldegrun, A., et al. (1989)
5   J. Immunol. **142**, 4520-4526; Topalian, S.L., et al. (1990) J. Immunol. **144**, 4487-4495; Nishimura, M.I., et al. (1993) J. Cell. Biochem. **17D**, 110. (Abstr.)). To determine whether multiple shared antigens were being recognized by CD4$^+$ TIL 1088, CD4$^+$ T cell clones were raised from these
10   TIL and assayed for target recognition. As shown in Table 1, 4 clones recognized the autologous melanoma as well as multiple allogeneic melanomas and all three normal melanocyte lines tested (FM 902, 906, 907). The target recognition profiles of all 4 clones were remarkably
15   similar and antibody blocking studies suggested that all were HLA-DR restricted. For 2 clones, HLA-DR restriction was confirmed by using allogeneic EBV-B cell lines or macrophages as APC for tumor antigen; only antigen presenting cells sharing the HLA-DR4 molecule were
20   stimulatory. Thus, a single antigenic protein seemed to be present in all of the melanomas recognized by these CD4$^+$ T cell clones, and was shared by normal melanocytes. Further experiments with these and 7 additional CD4$^+$ TIL 1088 clones revealed a homogeneous recognition profile,
25   suggesting the presence of an immunodominant epitope in the 1088 system.

CD4$^+$ TIL 1088 clones were assessed for recognition of melanoma-associated gene products expressed by 1088-mel on Northern blotting and known to contain
30   commonly expressed CD8 epitopes which can be recognized by CTL derived from melanoma patients. The genes encoding the tyrosinase, MART-1, and gp100 proteins (Brichard, V., et al. (1993) J. Exp. Med. **178**, 489-495; Kawakami, Y., et al. (1994) Proc. Natl. Acad. Sci. USA. **91**, 3515-3519;
35   Bakker, A.B.H., et al. (1994) J. Exp. Med. **179**, 1005-1009;

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Kawakami, Y., et al. (1994) *Proc. Natl. Acad. Sci. USA 91,
6458-6462)* were cloned into plasmid vectors and
transiently expressed in monkey kidney COS-7 cells.
Lysates of transfected COS-7 cells were pulsed onto 1088-
EBV and used to stimulate cytokine secretion from CD4^{+} T

5   cell clones. Table 2 shows that all 6 T-cell clones
tested secreted significant amounts of GM-CSF in response
to lysates of tyrosinase-transfected COS-7 cells as well
as to lysates from 1088-mel cells. Transfection of COS-7
cells with a truncated tyrosinase gene, or genes encoding

10   the gp75 tyrosinase-related protein, ß-galactosidase, or
HLA-A2.1 did not confer recognition. As a control, CD8^{+} T
cells from patient 1088 failed to react with any of these
stimulator cells. Of note, uncloned CD4^{+} T cells did not
react as strongly as the clones with the tyrosinase gene

15   product. This may reflect the presence of additional
epitopes recognized by CD4^{+} TIL 1088 subpopulations not
represented by these clones, as also suggested by some
discrepancies in the recognition of allogeneic melanomas
by the bulk-cultured TIL compared to the T cell clones

20   (compare Figure 2 to Table 1). In three additional
experiments, CD4^{+} T cell clones specifically recognized
the products of tyrosinase genes isolated from two
different patients' melanomas and expressed in two
different plasmids, while failing to react with MART-1 or

25   gp100 (not shown). Taken together with previous
demonstrations of CD8^{+} T cell reactivity in melanoma
patients against HLA-A2 and HLA-A24 restricted epitopes
encoded by the tyrosinase gene (Brichard, et al. (1993) *J.
Exp. Med.* 178:489-495; Robbins, P.F. et al. (1994) *Cancer*

30   *Research* 54:3124-3126), these findings show that a single
gene product, tyrosinase, contains epitopes recognized by
both CD4^{+} and CD8^{+} T lymphocytes. Furthermore, the fact
that the CD4^{+} TIL react to lysates of melanomas as well as
normal melanocytes suggests that the recognized epitope is

35   nonmutated.

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The importance of CD4$^+$ T cells in the priming and effector phases of the antitumor immune response has been shown in animal models (Greenberg, P.D., et al. (1985) *J. Exp. Med.* **161**, 1122-1134; Kern, D.E., et al. (1986) *J. Immunol.* **136**, 4303-4310; Ostrand-Rosenberg, S., Roby, C.A., & Clements, V.K. (1991) *J. Immunol.* **147**, 2419-2422; Dranoff, G., et al. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 3539-3543). Although experimental immunization strategies for patients with melanoma and other cancers currently emphasize shared class I restricted tumor antigens recognized by CD8$^+$ T cells, immunization against both class I and class II restricted epitopes may increase the effectiveness of these approaches.

Table 1.   CD4+ T cell clones recognize a shared antigen expressed on autologous and allogeneic melanomas and normal melanocytes

Stimulator*	GM-CSF secretion, † pg/ml per 24 hr (SI‡)			
	NT9	1D6	1B7	1E7
1088-mel	14,581 (504)	27,992 (384)	21,497 (169)	13,767 (101)
1011-mel	13,626 (471)	18,937 (260)	15,882 (125)	9,292 (68)
553-mel	2,856 (99)	11,067 (153)	9,357 (74)	4,777 (36)
697-mel	2,451 (86)	8,887 (123)	6,497 (52)	3,382 (26)
526-mel	168 (7)	2,117 (30)	1,400 (12)	196 (2)
624-mel	44 (3)	1,537 (22)	1,187 (10)	98 (2)
1087-mel	37 (2)	806 (12)	675 (6)	53 (1)
1290B-mel	<8 (1)	168 (3)	120 (2)	33 (1)
938-mel	<8 (1)	50 (2)	32 (1)	<8 (1)
1102-mel	<8 (1)	<8 (1)	<8 (1)	<8 (1)
586-mel	<8 (1)	55 (2)	18 (1)	<8 (1)
537-mel	<8 (1)	34 (1)	<8 (1)	<8 (1)
501A-mel	<8 (1)	24 (1)	<8 (1)	<8 (1)
677-mel	<8 (1)	22 (1)	<8 (1)	<8 (1)
CY13	<8 (1)	<8 (1)	<8 (1)	<8 (1)
1088-EBV	<8 (1)	<8 (1)	<8 (1)	<8 (1)
FM 902	5,831 (202)	12,667 (175)	10,517 (83)	6,557 (49)
FM 906	11,376 (393)	17,767 (244)	16,307 (128)	NT
FM 907	9,401 (325)	17,382 (239)	15,202 (120)	NT

NT, not tested.

* Cell lysates of cultured lines listed were incubated with 1088-EBV cells for 20 hr. In the same experiment, CD8+ TIL 1088 failed to react with these stimulators (data not shown).

† Net secretion = (secretion by TILs with EBV-B plus cell lysate) - (secretion by TILs with EBV-B). All cells were at 4 X 10⁴ per ml in microtiter plates.

‡ Stimulation index. GM-CSF secretion by TIL with EBV-B in the absence of cell lysate ranged from 29 to 138 pg/ml.

Table 2.   CD4+ T cell clones recognize a product of the tyrosinase gene

Stimulator*	Transfected gene†	GM-CSF secretion, ‡ pg/ml per 24 hr							
		CD4+ bulk TILs	CD8+ bulk TILs	NT9	1D6	1B7	1E7	2G2	2F9
1088-mel	None	6169	56	32,904	58,515	43,550	17,563	11,702	38,295
COS-7	Tyrosinase	123	<8	2,804	7,015	9,150	2,163	12,202	39,995
COS-7	Tyrosinase (1-3)	12	<8	<8	14	<8	<8	<8	<8
COS-7	Tyrosinase-related gp75	<8	<8	<8	<8	46	<8	<8	37
COS-7	β-Galactosidase	8	<8	<8	16	36	16	<8	<8
COS-7	HLA-A2.1	8	<8	<8	22	<8	<8	<8	12

* Cell lysates were cocultivated with 1088-EBV for 20 hr.

† Genes were expressed in the plasmid vector pcDNA3 except for the tyrosinase-related gp75 gene, which was in pCEV27.

‡ Net secretion is defined in footnote † to Table 1.   Values to TIL with EBV-B ranged from 10 to 237 pg/ml.

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Example II

MHC Class II Restricted Tyrosinase
Peptides And Modifications Thereof

MATERIALS AND METHODS

Peptide Synthesis. Peptides were synthesized
by a solid phase method using a peptide synthesizer, and
their molecular weights confirmed by mass spectrometry.
HA 307-319 and MT(65) 3-13, with high affinities for HLA-
DRB1*0401 and B1*0301, respectively, were synthesized for
use as inhibitors of tyrosinase peptide binding (Sette, A.
et al (1993) *J. Immunol.* 151, 3163-3170; Sidney J. et al
(1992) *J. Immunol.* 149, 2634-2640, herein incorporated by
reference).

Tumor Cell Lines And Other Cell Lines. Same as
in Example 1

Isolation of CD4$^+$ TIL 1088. Same as in Example
1.

Assessment of Antigen Recognition by CD4$^+$TIL
1088. EBV-B cells were incubated overnight in the
presence of peptides, up to 100μM concentrations. CD4$^+$ T
cells were added for an additional 24 hours, and cytokine
secretion measured to assess T cell stimulation (see
Example 1).

Results. Two tyrosinase peptides recognized by
bulk CD4$^+$ TIL1088 have been identified: Ty 56-70 and Ty
448-462 (Figure 7). This was done by screening
overlapping 15-mers based on a tyrosinase sequence derived
from melamona cell line 501-mel, (this sequence is the
same as GenBank J03581 sequence but with an R instead of Q
at amino acid position 402). These peptides are
nonmutated, since their amino acid sequences are identical
to a tyrosinase sequence derived from normal human
melanocytes (Kwon et. al., PNAS (1987) 84:7473-7477;
GenBank Accession Number J03581).

CD4$^+$ T cells from patient 1088 had been shown to
recognize lysates of autologous and some allogeneic

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melanoma cell lines expressing tyrosinase, and recognition
(measured via cytokine secretion) was HLA-DR-restricted
(Example 1; Figures 1 and 2). Patient 1088 was
heterozygous for DR, expressing HLA-DRB1*0301 and B1*0401.
A peptide binding HLA-DRB1*0401 with high affinity, HA
5   307-319, effectively blocked binding of both the Ty 56-70
and Ty 448-462 peptides to the presenting EBV-B cells
derived from patient 1088, leading to decreased T cell
recognition (see Figure 4). A peptide with high affinity
for HLA-DRB1*0301, MT(65)3-13, failed to inhibit T cell
10   recognition. Neither HA 307-319 nor MT(65) 3-13 affected
T cell recognition of a melanoma lysate, which is
internalized by B cells and processed prior to
presentation (Topalian Int. J. Cancer, 1994 58:69-79).
The HLA-restriction of Ty 56-70 and Ty 448-462 was
15   confirmed as HLA-DRB1*0401 by using B cell lines of
various HLA genotypes as antigen presenting cells (See
Figure 5).

In order to make more immunogenic peptides for
induction of a CD4^{+} T cell response, a variety of peptide
20   epitopes were synthesized in which at least one amino acid
position was changed based on the binding motifs of
peptides presented by HLA-DRB*0401 (Sette, A. et al (1993)
J. Immunol. 151, 3163-3170; Rammensee, H.G. (1995)
Immunogenetics 41, 178-228, herein incorporated by
25   reference). The P1 anchor position of Ty 56-70 was
hypothesized to be I58, L59, or L60 and the P6 anchor to
be A63, P64 or L65 (Figure 6). To confirm this, peptides
with amino acid substitutions at these positions (Q =
unfavorable substitution, F or V = favorable) and serially
30   truncated peptides were tested for T cell recognition.
The results shown in Figure 6 suggest that I58 and A63 are
the P1 and P6 anchors, respectively, of Ty 56-70 (Figure
7). In addition, two modified peptides (Ty 56-70, A63 → V
and Ty 56-70, L65 → V) seemed to evoke an enhanced T cell
35   response compared to the unmodified Ty 56-70. This was

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confirmed by titrating the T cell response to these peptides (Figure 8).

In repeated experiments, Ty 448-462 was specifically recognized by CD4$^+$ T cells from patient 1088. By testing modified peptides with either unfavorable (Q) or favorable (F) amino acid substitutions at potential P1 anchor positions (Figure 9), and by testing a series of truncated peptides, Y451 was identified as the P1 anchor. At this position, the unfavorable amino acid substitution abolished T cell recognition, while the favorable substitution restored it. In addition, it was found that the truncated Ty 449-462 and 450-462 peptides were more stimulatory for T cells than the parent peptide. This was confirmed in a subsequent titration experiment (see Figure 10).

The P6 anchor position for Ty 448-462 was presumed to be D456, based on identifying Y451 as the P1 anchor.D (aspartic acid) is not an optimal residue at this position, and a valine substitution led to markedly enhanced T cell recognition (see Figure 11). When two favorable modifications of Ty 448-462 were combined in a single modified peptide (Ty 450-462, D456→V), recognition was enhanced even more (see Figure 12).

In summary, both Ty 56-70 and Ty 448-462 are restricted by HLA-DRB1*0401. This MHC molecule is expressed by approximately 15% of the North American Caucasian population. The anchor positions in Ty 56-70 and Ty 448-462 have been identified (see Figure 7), and substitutions of amino acids at these positions have created modified peptides with enhanced T cell stimulatory properties.

The utility of these peptides in the prophylaxis and/or therapy of melanoma may not be limited to patients expressing the Class II MHC molecule DRB1*0401, as Class II-restricted peptides are often capable of binding to more than one Class II molecule (Chicz, R.M. et al (1993)

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J. *Exp. Med.* 178, 27-47; Malcherek, G. et al (1995) *J. Exp. Med.* 181, 527-536).

Although the present invention has been described in some detail by way of illustration, and examples for purposes of clarification and understanding it will be obvious that certain changes may be made within the scope of the appended claims. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i)   APPLICANTS: THE GOVERNMENT OF THE UNITED
             STATES OF AMERICA AS REPRESENTED BY THE
             SECRETARY, DEPARTMENT OF HEALTH AND HUMAN
             SERVICES

    (ii)   TITLE OF INVENTION: MHC-CLASS II
             RESTRICTED MELANOMA ANTIGENS AND THEIR USE IN
             DIAGNOSTIC AND THERAPEUTIC METHODS

    (iii)   NUMBER OF SEQUENCES: 38

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    (v)   COMPUTER READABLE FORM:
             (A)   MEDIUM TYPE: FLOPPY DISK
             (B)   COMPUTER: IBM PC COMPATIBLE
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    (vi)   CURRENT APPLICATION DATA:
             (A)   APPLICATION NUMBER: TO BE ASSIGNED
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             (A)   APPLICATION NUMBER: 08/533,895
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    (viii) ATTORNEY/AGENT INFORMATION:
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             (C)   TELEX: 421792

(2) INFORMATION FOR SEQ ID NO:1:

    (i)   SEQUENCE CHARACTERISTICS:
             (A)   LENGTH: 15

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```
                    (B)   TYPE:  AMINO ACID
                    (C)   STRANDEDNESS:  UNKNOWN
                    (D)   TOPOLOGY:  UNKNOWN

          (xi)    SEQUENCE DESCRIPTION:  SEQ ID NO:1:

Gln Asn Ile Leu Leu Ser Asn Ala Pro Leu Gly Pro
  1               5                  10

Gln Phe Pro
        15



    (2)   INFORMATION FOR SEQ ID NO:2:

          (i)     SEQUENCE CHARACTERISTICS:
                  (A)   LENGTH:  14
                  (B)   TYPE:  AMINO ACID
                  (C)   STRANDEDNESS:  UNKNOWN
                  (D)   TOPOLOGY:  UNKNOWN

          (xi)    SEQUENCE DESCRIPTION:  SEQ ID NO:2:

Asn Ile Leu Leu Ser Asn Ala Pro Leu Gly Pro Gln
  1               5                  10

Phe Pro



    (2)   INFORMATION FOR SEQ ID NO:3:

          (i)     SEQUENCE CHARACTERISTICS:
                  (A)   LENGTH:  15
                  (B)   TYPE:  AMINO ACID
                  (C)   STRANDEDNESS:  UNKNOWN
                  (D)   TOPOLOGY:  UNKNOWN

          (xi)    SEQUENCE DESCRIPTION:  SEQ ID NO:3:

Gln Asn Ile Leu Leu Ser Asn Ala Pro Val Gly Pro
  1               5                  10

Gln Phe Pro
        15


    (2)   INFORMATION FOR SEQ ID NO:4:

          (i)     SEQUENCE CHARACTERISTICS:
                  (A)   LENGTH:  15
                  (B)   TYPE:  AMINO ACID
                  (C)   STRANDEDNESS:  UNKNOWN

                  (D)   TOPOLOGY:  UNKNOWN
```

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(xi)   SEQUENCE DESCRIPTION: SEQ ID NO:4:

Gln Asn Ile Leu Leu Ser Asn Val Pro Val Gly Pro
 1   5   10

Gln Phe Pro
        15

(2)   INFORMATION FOR SEQ ID NO:5:

     (i)   SEQUENCE CHARACTERISTICS:
                (A)   LENGTH: 15
                (B)   TYPE: AMINO ACID
                (C)   STRANDEDNESS: UNKNOWN
                (D)   TOPOLOGY: UNKNOWN

     (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:5:

Gln Asn Ile Leu Leu Ser Asn Val Pro Leu Gly Pro
 1   5   10

Gln Phe Pro
        15

(2)   INFORMATION FOR SEQ ID NO:6:

     (i)   SEQUENCE CHARACTERISTICS:
                (A)   LENGTH: 15
                (B)   TYPE: AMINO ACID
                (C)   STRANDEDNESS: UNKNOWN
                (D)   TOPOLOGY: UNKNOWN

     (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:6:

Asp Tyr Ser Tyr Leu Gln Asp Ser Asp Pro Asp Ser
 1   5   10

Phe Gln Asp
        15

(2)   INFORMATION FOR SEQ ID NO:7:

     (i)   SEQUENCE CHARACTERISTICS:
                (A)   LENGTH: 15
                (B)   TYPE: AMINO ACID
                (C)   STRANDEDNESS: UNKNOWN
                (D)   TOPOLOGY: UNKNOWN

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(xi)   SEQUENCE DESCRIPTION: SEQ ID NO:7:

Asp Tyr Ser Tyr Leu Gln Asp Ser Asp Pro Asp Ser
 1   5   10

Ser Gln Asp
         15

(2)   INFORMATION FOR SEQ ID NO:8:

    (i)   SEQUENCE CHARACTERISTICS:
               (A)   LENGTH: 15
               (B)   TYPE: AMINO ACID
               (C)   STRANDEDNESS: UNKNOWN
               (D)   TOPOLOGY: UNKNOWN

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:8:

Asp Gln Ser Tyr Leu Gln Asp Ser Asp Pro Asp Ser
 1   5   10

Phe Gln Asp
         15

(2)   INFORMATION FOR SEQ ID NO:9:

    (i)   SEQUENCE CHARACTERISTICS:
               (A)   LENGTH: 15
               (B)   TYPE: AMINO ACID
               (C)   STRANDEDNESS: UNKNOWN
               (D)   TOPOLOGY: UNKNOWN

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:9:

Asp Phe Ser Tyr Leu Gln Asp Ser Asp Pro Asp Ser
 1   5   10

Phe Gln Asp
         15

(2)   INFORMATION FOR SEQ ID NO:10:

    (i)   SEQUENCE CHARACTERISTICS:
               (A)   LENGTH: 15
               (B)   TYPE: AMINO ACID
               (C)   STRANDEDNESS: UNKNOWN
               (D)   TOPOLOGY: UNKNOWN

(xi)   SEQUENCE DESCRIPTION: SEQ ID NO:10:

Asp Tyr Ser Phe Leu Gln Asp Ser Asp Pro Asp Ser
 1   5   10

Phe Gln Asp
        15

(2)   INFORMATION FOR SEQ ID NO:11:

    (i)   SEQUENCE CHARACTERISTICS:
             (A)   LENGTH: 15
             (B)   TYPE: AMINO ACID
             (C)   STRANDEDNESS: UNKNOWN
             (D)   TOPOLOGY: UNKNOWN

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:11:

Asp Tyr Ser Tyr Leu Gln Asp Ser Val Pro Asp Ser
 1   5   10

Phe Gln Asp
        15

(2)   INFORMATION FOR SEQ ID NO:12:

    (i)   SEQUENCE CHARACTERISTICS:
             (A)   LENGTH: 13
             (B)   TYPE: AMINO ACID
             (C)   STRANDEDNESS: UNKNOWN
             (D)   TOPOLOGY: UNKNOWN

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:12:

Ser Tyr Leu Gln Asp Ser Val Pro Asp Ser Phe Gln
 1   5   10

Asp

(2)   INFORMATION FOR SEQ ID NO:13:

    (i)   SEQUENCE CHARACTERISTICS:
             (A)   LENGTH: 14
             (B)   TYPE: AMINO ACID
             (C)   STRANDEDNESS: UNKNOWN
             (D)   TOPOLOGY: UNKNOWN

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(xi)   SEQUENCE DESCRIPTION: SEQ ID NO:13:

Tyr Ser Tyr Leu Gln Asp Ser Asp Pro Asp Ser Phe
 1   5   10

Gln Asp

(2)   INFORMATION FOR SEQ ID NO:14:

      (i)   SEQUENCE CHARACTERISTICS:
               (A)   LENGTH: 13
               (B)   TYPE: AMINO ACID
               (C)   STRANDEDNESS: UNKNOWN
               (D)   TOPOLOGY: UNKNOWN

      (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:14:

Ser Tyr Leu Gln Asp Ser Asp Pro Asp Ser Phe Gln
 1   5   10

Asp

(2)   INFORMATION FOR SEQ ID NO:15:

      (i)   SEQUENCE CHARACTERISTICS:
               (A)   LENGTH: 9
               (B)   TYPE: AMINO ACID
               (C)   STRANDEDNESS: UNKNOWN
               (D)   TOPOLOGY: UNKNOWN

      (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:15:

Xaa Leu Leu Xaa Asn Xaa Xaa Leu Xaa
 1   5

(2)   INFORMATION FOR SEQ ID NO:16:

      (i)   SEQUENCE CHARACTERISTICS:
               (A)   LENGTH: 9
               (B)   TYPE: AMINO ACID
               (C)   STRANDEDNESS: UNKNOWN
               (D)   TOPOLOGY: UNKNOWN

      (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:16:

Xaa Leu Gln Xaa Ser Xaa Xaa Asp Xaa
 1   5

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(2)   INFORMATION FOR SEQ ID NO:17:

    (i)   SEQUENCE CHARACTERISTICS:
          (A)   LENGTH: 15
          (B)   TYPE: AMINO ACID
          (C)   STRANDEDNESS: UNKNOWN
          (D)   TOPOLOGY: UNKNOWN

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:17:

```
Gln Asn Gln Leu Leu Ser Asn Ala Pro Leu Gly Pro
  1               5                   10

Gln Phe Pro
        15
```

(2)   INFORMATION FOR SEQ ID NO:18:

    (i)   SEQUENCE CHARACTERISTICS:
          (A)   LENGTH: 15
          (B)   TYPE: AMINO ACID
          (C)   STRANDEDNESS: UNKNOWN
          (D)   TOPOLOGY: UNKNOWN

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:18:

```
Gln Asn Phe Leu Leu Ser Asn Ala Pro Leu Gly Pro
  1               5                   10

Gln Phe Pro
        15
```

(2)   INFORMATION FOR SEQ ID NO:19:

    (i)   SEQUENCE CHARACTERISTICS:
          (A)   LENGTH: 15
          (B)   TYPE: AMINO ACID
          (C)   STRANDEDNESS: UNKNOWN
          (D)   TOPOLOGY: UNKNOWN

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:19:

```
Gln Asn Val Leu Leu Ser Asn Ala Pro Leu Gly Pro
  1               5                   10

Gln Phe Pro
        15
```

(2)   INFORMATION FOR SEQ ID NO:20:

    (i)   SEQUENCE CHARACTERISTICS:
          (A)   LENGTH: 15

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```
                        (B)   TYPE:  AMINO ACID
                        (C)   STRANDEDNESS:  UNKNOWN
                        (D)   TOPOLOGY:  UNKNOWN

        (xi)      SEQUENCE DESCRIPTION: SEQ ID NO:20:

Gln Asn Ile Gln Leu Ser Asn Ala Pro Leu Gly Pro
 1               5                   10

Gln Phe Pro
        15


    (2)   INFORMATION FOR SEQ ID NO:21:

        (i)       SEQUENCE CHARACTERISTICS:
                        (A)   LENGTH:  15
                        (B)   TYPE:  AMINO ACID
                        (C)   STRANDEDNESS:  UNKNOWN
                        (D)   TOPOLOGY:  UNKNOWN

        (xi)      SEQUENCE DESCRIPTION: SEQ ID NO:21:

Gln Asn Ile Phe Leu Ser Asn Ala Pro Leu Gly Pro
 1               5                   10

Gln Phe Pro
        15


    (2)   INFORMATION FOR SEQ ID NO:22:

        (i)       SEQUENCE CHARACTERISTICS:
                        (A)   LENGTH:  15
                        (B)   TYPE:  AMINO ACID
                        (C)   STRANDEDNESS:  UNKNOWN
                        (D)   TOPOLOGY:  UNKNOWN

        (xi)      SEQUENCE DESCRIPTION: SEQ ID NO:22:

Gln Asn Ile Val Leu Ser Asn Ala Pro Leu Gly Pro
 1               5                   10

Gln Phe Pro
        15


    (2)   INFORMATION FOR SEQ ID NO:23:

        (i)       SEQUENCE CHARACTERISTICS:
                        (A)   LENGTH:  15
                        (B)   TYPE:  AMINO ACID
                        (C)   STRANDEDNESS:  UNKNOWN
                        (D)   TOPOLOGY:  UNKNOWN
```

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    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:23:

Gln Asn Ile Leu Gln Ser Asn Ala Pro Leu Gly Pro
  1   5   10

Gln Phe Pro
        15

    (2) INFORMATION FOR SEQ ID NO:24:

        (i)   SEQUENCE CHARACTERISTICS:
                  (A)   LENGTH: 15
                  (B)   TYPE: AMINO ACID
                  (C)   STRANDEDNESS: UNKNOWN
                  (D)   TOPOLOGY: UNKNOWN

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:24:

Gln Asn Ile Leu Phe Ser Asn Ala Pro Leu Gly Pro
  1   5   10

Gln Phe Pro
        15

    (2) INFORMATION FOR SEQ ID NO:25:

        (i)   SEQUENCE CHARACTERISTICS:
                  (A)   LENGTH: 15
                  (B)   TYPE: AMINO ACID
                  (C)   STRANDEDNESS: UNKNOWN
                  (D)   TOPOLOGY: UNKNOWN

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:25:

Gln Asn Ile Leu Val Ser Asn Ala Pro Leu Gly Pro
  1   5   10

Gln Phe Pro
        15

    (2) INFORMATION FOR SEQ ID NO:26:

        (i)   SEQUENCE CHARACTERISTICS:
                  (A)   LENGTH: 15
                  (B)   TYPE: AMINO ACID
                  (C)   STRANDEDNESS: UNKNOWN
                  (D)   TOPOLOGY: UNKNOWN

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(xi)   SEQUENCE DESCRIPTION: SEQ ID NO:26:

Gln Asn Ile Leu Leu Ser Asn Gln Pro Leu Gly Pro
 1   5   10

Gln Phe Pro
        15

(2)   INFORMATION FOR SEQ ID NO:27:

     (i)   SEQUENCE CHARACTERISTICS:
               (A)   LENGTH: 15
               (B)   TYPE: AMINO ACID
               (C)   STRANDEDNESS: UNKNOWN
               (D)   TOPOLOGY: UNKNOWN

     (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:27:

Gln Asn Ile Leu Leu Ser Asn Ala Gln Leu Gly Pro
 1   5   10

Gln Phe Pro
        15

(2)   INFORMATION FOR SEQ ID NO:28:

     (i)   SEQUENCE CHARACTERISTICS:
               (A)   LENGTH: 15
               (B)   TYPE: AMINO ACID
               (C)   STRANDEDNESS: UNKNOWN
               (D)   TOPOLOGY: UNKNOWN

     (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:28:

Gln Asn Ile Leu Leu Ser Asn Ala Val Leu Gly Pro
 1   5   10

Gln Phe Pro
        15

(2)   INFORMATION FOR SEQ ID NO:29:

     (i)   SEQUENCE CHARACTERISTICS:
               (A)   LENGTH: 15
               (B)   TYPE: AMINO ACID
               (C)   STRANDEDNESS: UNKNOWN
               (D)   TOPOLOGY: UNKNOWN

     (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:29:

Gln Asn Ile Leu Leu Ser Asn Ala Pro Gln Gly Pro
 1   5   10

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Gln Phe Pro
          15

    (2)   INFORMATION FOR SEQ ID NO:30:

        (i)   SEQUENCE CHARACTERISTICS:
                  (A)   LENGTH: 13
                  (B)   TYPE: AMINO ACID
                  (C)   STRANDEDNESS: UNKNOWN
                  (D)   TOPOLOGY: UNKNOWN

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:30:

Ile Leu Leu Ser Asn Ala Pro Leu Gly Pro Gln Phe
  1   5   10

Pro

    (2)   INFORMATION FOR SEQ ID NO:31:

        (i)   SEQUENCE CHARACTERISTICS:
                  (A)   LENGTH: 13
                  (B)   TYPE: AMINO ACID
                  (C)   STRANDEDNESS: UNKNOWN
                  (D)   TOPOLOGY: UNKNOWN

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:31:

Leu Leu Ser Asn Ala Pro Leu Gly Pro Gln Phe Pro Phe
  1   5   10

    (2)   INFORMATION FOR SEQ ID NO:32:

        (i)   SEQUENCE CHARACTERISTICS:
                  (A)   LENGTH: 13
                  (B)   TYPE: AMINO ACID
                  (C)   STRANDEDNESS: UNKNOWN
                  (D)   TOPOLOGY: UNKNOWN

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:32:

Leu Ser Asn Ala Pro Leu Gly Pro Gln Phe Pro Phe Thr
  1   5   10

    (2)   INFORMATION FOR SEQ ID NO:33:

        (i)   SEQUENCE CHARACTERISTICS:
                  (A)   LENGTH: 15
                  (B)   TYPE: AMINO ACID

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```
                        (C)   STRANDEDNESS:   UNKNOWN
                        (D)   TOPOLOGY:   UNKNOWN

              (xi)      SEQUENCE DESCRIPTION: SEQ ID NO:33:

     Ser Asn Ala Pro Leu Gly Pro Gln Phe Pro Phe
      1               5                   10

     Thr Gly Val Asp
                 15



     (2)   INFORMATION FOR SEQ ID NO:34:

              (i)       SEQUENCE CHARACTERISTICS:
                        (A)   LENGTH:   15
                        (B)   TYPE:   AMINO ACID
                        (C)   STRANDEDNESS:   UNKNOWN
                        (D)   TOPOLOGY:   UNKNOWN

              (xi)      SEQUENCE DESCRIPTION: SEQ ID NO:34:

     Asp Tyr Ser Gln Leu Gln Asp Ser Asp Pro Asp Ser
      1               5                   10

     Phe Gln Asp
             15



     (2)   INFORMATION FOR SEQ ID NO:35:

              (i)       SEQUENCE CHARACTERISTICS:
                        (A)   LENGTH:   15
                        (B)   TYPE:   AMINO ACID
                        (C)   STRANDEDNESS:   UNKNOWN
                        (D)   TOPOLOGY:   UNKNOWN

              (xi)      SEQUENCE DESCRIPTION: SEQ ID NO:35:

     Asp Tyr Ser Tyr Gln Gln Asp Ser Asp Pro Asp Ser
      1               5                   10

     Phe Gln Asp
             15


     (2)   INFORMATION FOR SEQ ID NO:36:

              (i)       SEQUENCE CHARACTERISTICS:
                        (A)   LENGTH:   15
                        (B)   TYPE:   AMINO ACID
                        (C)   STRANDEDNESS:   UNKNOWN
                        (D)   TOPOLOGY:   UNKNOWN
```

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(xi)   SEQUENCE DESCRIPTION: SEQ ID NO:36:

Asp Tyr Ser Tyr Phe Gln Asp Ser Asp Pro Asp Ser
 1   5   10

Phe Gln Asp
        15

(2)   INFORMATION FOR SEQ ID NO:37:

    (i)   SEQUENCE CHARACTERISTICS:
              (A)   LENGTH: 12
              (B)   TYPE: AMINO ACID
              (C)   STRANDEDNESS: UNKNOWN
              (D)   TOPOLOGY: UNKNOWN

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:37:

Tyr Leu Gln Asp Ser Asp Pro Asp Ser Phe Gln Asp
 1   5   10

(2)   INFORMATION FOR SEQ ID NO:38:

    (i)   SEQUENCE CHARACTERISTICS:
              (A)   LENGTH: 11
              (B)   TYPE: AMINO ACID
              (C)   STRANDEDNESS: UNKNOWN
              (D)   TOPOLOGY: UNKNOWN

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:38:

Leu Gln Asp Ser Asp Pro Asp Ser Phe Gln Asp
 1   5   10

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We Claim:

     1.   An isolated MHC Class II immunogenic peptide comprising at least 9 contiguous amino acids of a MHC Class II core binding sequence from a tyrosinase sequence.

     2.   The immunogenic peptide of claim 1 where said peptide contains a sequence selected from the group consisting of QNILLSNAPLGPQFP (Ty 56-70), NILLSNAPLGPQFP (Ty 57-70), DYSYLQDSDPDSFQD (Ty 448-462), YSYLQDSDPDSFQD (Ty 449-462), and SYLQDSDPDSFQD (Ty 450-462).

     3.   The immunogenic peptide of claim 2 having at least 9 contiguous residues, wherein said peptide has 1,2, 3, 4 or 5 amino acid deletions at the carboxy or amino terminus and maintains requisite activity.

     4.   The immunogenic peptide of claim 1, wherein said peptide sequence contains at least one amino acid modification of said tyrosinase sequence to enhance binding of peptide to an MHC Class II molecule or to a T cell receptor.

     5.   The immunogenic peptide of Claim 4 containing a sequence selected from the group consisting of QNILLSNAPVGPQFP (Ty 56-70, L65→V), QNILLSNVPVGPQFP (Ty 56-70, A63→V and L65→V), QNILLSNVPLGPQFP (Ty 56-70, A63→V), DYSYLQDSDPDSSQD (Ty 448-462, F460→S), DQSYLQDSDPDSFQD (Ty 448-462, Y449-Q), DFSYLQDSDPDSFQD (Ty 448-462, Y449→F), DYSFLQDSDPDSFQD (Y451→F), and DYSYLQDSVPDSFQD (Ty 448-462, D456→V).

     6.   The immunogenic peptide of claim 5 having at least 9 contiguous residues of claim 2, wherein said peptide has 1,2, 3, 4 or 5 amino acid deletions at the carboxy or amino terminus and maintains requisite activity.

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7.   The immunogenic peptide of claim 4 wherein an amino acid substitution is within a 9 amino acid Class II binding core sequence in said peptide.

8.   The peptide of claim 7, wherein said amino acid substitution is located at a position selected from the group consisting of: (i) the first position, (ii) the fourth position, (iii) the sixth position, (iv) the seventh position, (v) the ninth position and (vi) combinations of at least two of (i) - (v) in the sequence of the core binding region of the peptide.

9.   The peptide of claim 6, wherein said amino acid substitution are located at the first and sixth positions.

10.   An immunogenic peptide having the core binding sequence formula $X_1LLX_2NX_3X_4LX_5$ or $X_1LQX_2SX_3X_4DX_5$ wherein:

X_1 is any hydrophobic amino acid;

X_2 is any hydrophobic amino acid; aspartic acid, or glutamic acid;

X_3 is any hydrophobic or hydroxyl amino acid;

X_4 is any polar, charged or aliphatic amino acid; and

X_5 is any polar or aliphatic amino acid.

11.   The peptide of claim 10 wherein the peptide is lengthened by flanking regions at the amino and/or carboxy termini to a total of 34 amino acids.

12.   The peptide of claim 10, wherein X_1 is selected from the group consisting of methionine, leucine, isoleucine, tyrosine, valine, tryptophan and phenylalanine.

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13.   The peptide of claim 10, wherein X_2 is selected from the group consisting of phenylalanine, tryptophan, leucine, isoleucine, alanine, valine, aspartic acid and glutamic acid.

14.   The peptide of claim 10, wherein X_3 is selected from the group consisting of methionine, leucine, threonine, isoleucine, serine and valine.

15.   The peptide of claim 10, wherein X_4 is selected from the group consisting of aspartic acid, alanine, serine, valine, histidine, proline, asparginine, methionine, threonine, leucine and isoleucine.

16.   The peptide of claim 10, wherein X_5 is selected from the group consisting of alanine, serine, glutamine, glycine, leucine, valine and threonine.

17.   The immunogenic peptide of claim 1 wherein said peptide is recognized by HLA-DR $CD4^+$ T lymphocytes.

18.   The immunogenic peptide of claim 10 wherein said peptide is recognized by HLA-DR CD4+ T lymphocytes.

19.   The immunogenic peptide of claim 1 linked to a MHC Class II molecule.

20.   The immunogenic peptide of claim 10 linked to a MHC Class II molecule.

21.   The immunogenic peptide-MHC complex of claim 19 wherein said MHC Class II molecule is the β chain of the MHC Class II molecule.

22.   The immunogenic peptide-MHC complex of claim 20 wherein said MHC Class II molecule is the β chain of the MHC Class II molecule.

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     23. A pharmaceutical composition comprising at least one peptide of claim 1, and an acceptable excipient, diluent or carrier.

     24. A pharmaceutical composition comprising at least one peptide of claim 10, and an acceptable excipient, diluent or carrier.

     25. A pharmaceutical composition comprising at least one peptide of claim 19, and an acceptable excipient, diluent or carrier.

     26. A pharmaceutical composition comprising at least one peptide of claim 20, and an acceptable excipient, diluent or carrier.

     27. A method of preventing or treating melanoma comprising administering a therapeutically effective amount of the the pharmaceutical composition of claim 23 to a mammal in an effective amount to stimulate the production of protective antibodies or immune cells.

     28. A method of preventing or treating melanoma comprising administering a therapeutically effective amount of the the pharmaceutical composition of claim 24 to a mammal in an effective amount to stimulate the production of protective antibodies or immune cells.

     29. A method of preventing or treating melanoma comprising administering a therapeutically effective amount of the the pharmaceutical composition of claim 25 to a mammal in an effective amount to stimulate the production of protective antibodies or immune cells.

     30. A method of preventing or treating melanoma comprising administering a therapeutically effective amount of the the pharmaceutical composition of claim 26

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to a mammal in an effective amount to stimulate the
production of protective antibodies or immune cells.

        31.   A purified and isolated nucleic acid
sequence encoding a peptide according to claim 1.

        32.   A purified and isolated nucleic acid
sequence encoding a peptide according to claim 10.

        33.   A purified and isolated nucleic acid
sequence encoding a peptide according to claim 19.

        34.   A purified and isolated nucleic acid
sequence encoding a peptide or polypeptide according to
claim 20.

        35.   A recombinant expression vector comprising
at least one nucleic acid sequence of claim 1.

        36.   A recombinant expression vector comprising
at least one nucleic acid sequence claim 10.

        37.   A recombinant expression vector comprising
at least one nucleic acid sequence of claim 19.

        38.   A recombinant expression vector comprising
at least one nucleic acid sequence of claim 20.

        39.   The vector of claim 35, wherein the
expression vector is a eukaryotic expression vector or
prokaryotic expression vector.

        40.   The vector of claim 36, wherein the
expression vector is a eukaryotic expression vector or
prokaryotic expression vector.

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41.   The vector of claim 37, wherein the expression vector is a eukaryotic expression vector or prokaryotic expression vector.

42.   The vector of claim 38, wherein the expression vector is a eukaryotic expression vector or prokaryotic expression vector.

43.   A host cell containing with the recombinant expression vector according to claim 39.

44.   A host cell containing with the recombinant expression vector according to claim 40.

45.   A host cell containing with the recombinant expression vector according to claim 41.

46.   A host cell containing with the recombinant expression vector according to claim 42.

47.   Antibodies reactive with an immunogenic peptide of claim 1.

48.   Antibodies reactive with an immunogenic peptide of claim 10.

49.   Antibodies reactive with an immunogenic peptide of claim 19.

50.   Antibodies reactive with an immunogenic peptide of claim 20.

51.   The antibodies of claim 48 wherein said antibodies are monoclonal.

52.   A therapeutic method of preventing or treating melanoma comprising administering the

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pharmaceutical composition of claim 23 to a mammal in an effective amount to stimulate the production of protective antibodies or immune positive CD4$^+$ T-cells.

53.   A therapeutic method of preventing or treating melanoma comprising administering the pharmaceutical composition of claim 24 to a mammal in an effective amount to stimulate the production of protective antibodies or immune positive CD4$^+$ T-cells.

54.   A therapeutic method of preventing or treating melanoma comprising administering the pharmaceutical composition of claim 25 to a mammal in an effective amount to stimulate the production of protective antibodies or immune positive CD4$^+$ T-cells.

55.   A therapeutic method of preventing or treating melanoma comprising administering the pharmaceutical composition of claim 26 to a mammal in an effective amount to stimulate the production of protective antibodies or immune positive CD4$^+$ T-cells.

56.   The method of claim 52, wherein said composition includes isolated tyrosine protein.

57.   A method for determining or isolating Class II tumor associated antigens, said method comprising the steps of:
            (a)   contacting a candidate antigen with antigen presenting cells for a time sufficient to allow the antigen to be processed by said antigen presenting cells;
            (b)   contacting said antigen presenting cells step (a) with CD4$^+$ T lymphocytes; and
            (c)   screening for recognition of said antigen presenting cells by said CD4$^+$ T lymphocytes.

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58. The method of claim 57 wherein said candidate antigen is selected from the group consisting of a crude cellular lysate, a purified protein, a peptide, or proteins encoding by a DNA library.

5   59. The method of claim 57 wherein said antigen presenting cells are EBV transformed B cells, monocytes or dendritic cells.

60. The method of claim 57 wherein said
10   candidate antigen is selected from the group consisting of MART-1, gp100, gp-75, MAGE-1, MAGE-3, or p15 or any other tumor associated protein known to be recognized by $CD8^+T$ cells via MHC Class I restriction.

15   61. The melanoma antigens or tumor associated antigens isolated by screening candidate antigens according to claim 57.

62. A purified and isolated nucleic acid
20   sequence encoding a peptide comprising at least about 9 contiguous amino acids, said peptide being derived from a tyrosinase sequence, said peptide being reactive to $CD4^+$ T-lymphocytes.

25   63. A recombinant expression vector comprising at least one nucleic acid sequence of claim 62.

64. The use of the peptides of claims 1-22 in the manufacture of a medicament for stimulating the
30   production of protective antibodies or immune cells in a mammal.

65. The use of the peptides of claims 1-22 in the manufacture of a medicament for preventing or treating
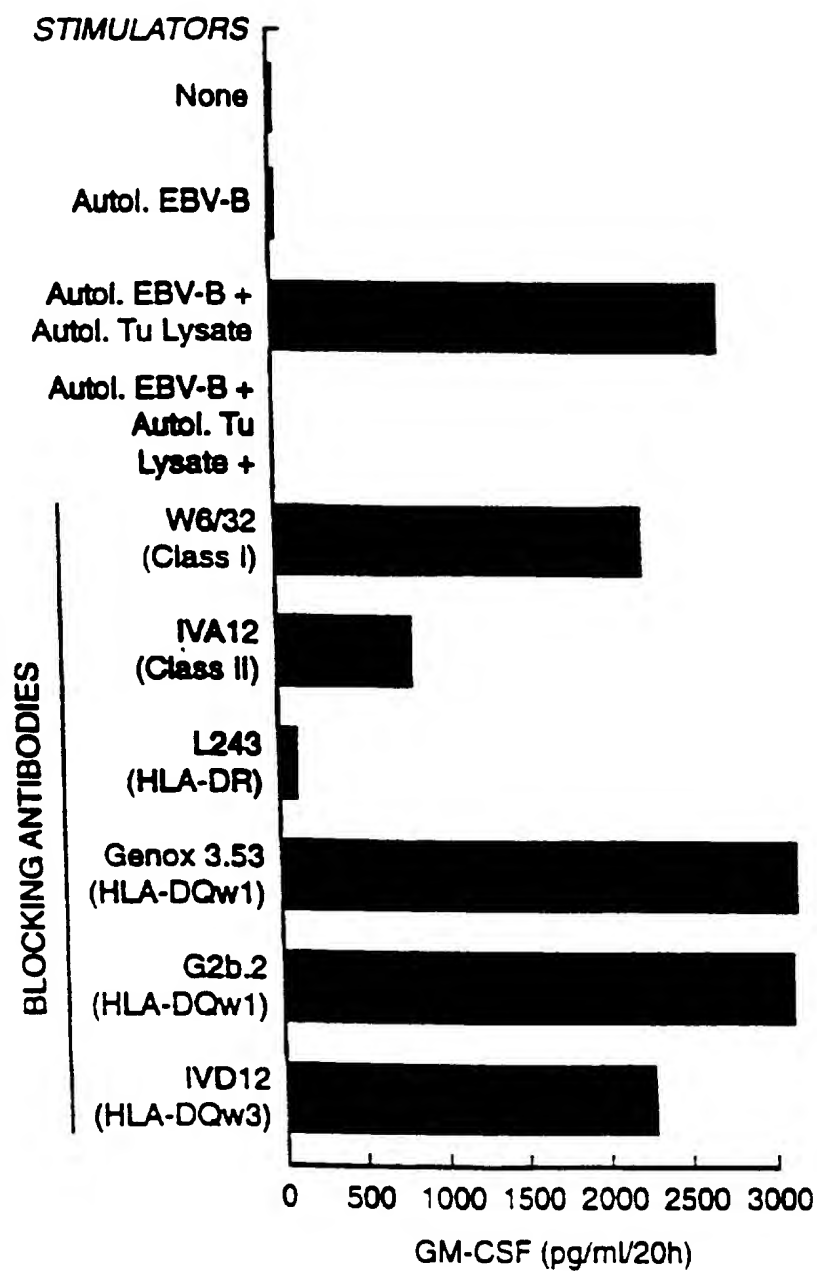35   melanoma by stimulating the production of protective antibodies or immune positive $CD4^+$ T-cells.
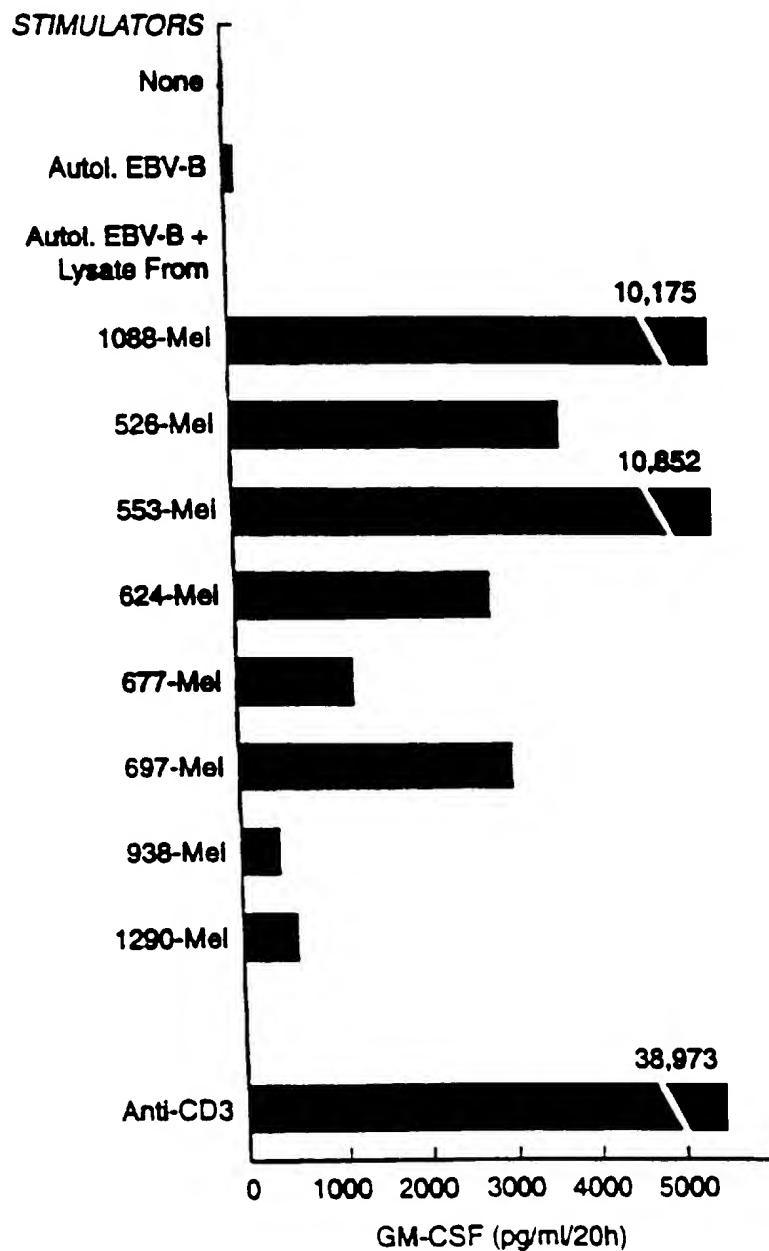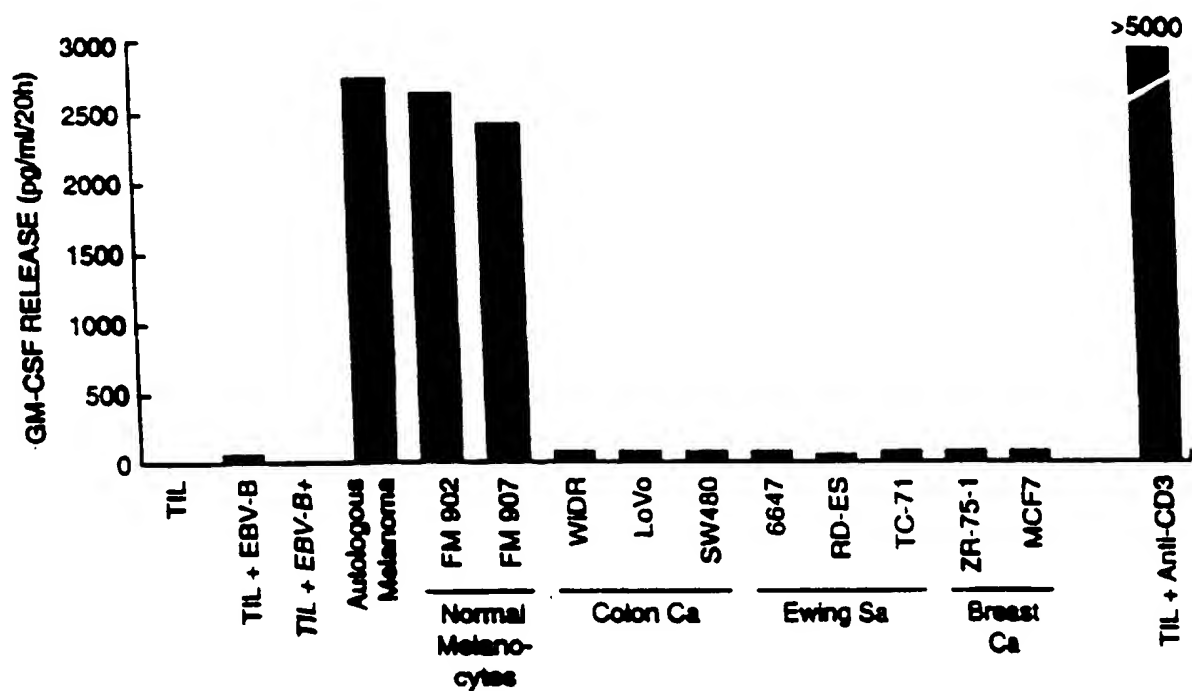
FIGURE 1

FIGURE 2

FIGURE 3

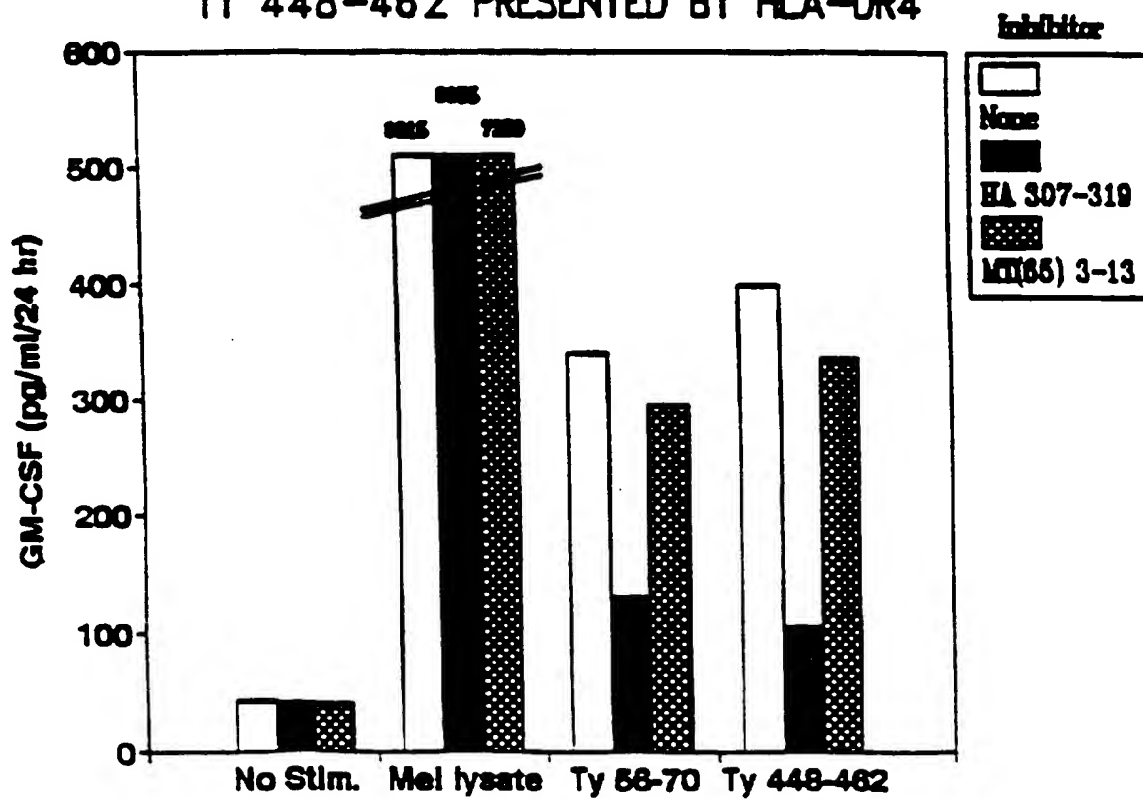FIGURE 4

HLA Restriction of Tyrosinase Recognition

by CD4° TIL 1088

(GM-CSF secretion, pg/ml/24 hr)

Antigen Presenting Cells	Antigen Presented				HLA-DRβ1°	
	None	1088-mel lysate	Ty 56-70 (A63→V)	Ty 448-462 (D456→V)		
Experiment 1						
1088-EBV	145	1179	2445	2969	0301	0401
697-EBV	282	1014	>500	3815	0401	1501
BM14	751	3282	4793	5121	0401	-
860-EBV	376	870	383	345	0301	1301
583-EBV	484	550	574	477	0404	1501
1087-EBV	507	557	550	566	0701	1201
Experiment 2						
1389-EBV	183	2238	1179	1714	0301	0401
BM14	372	2822	1892	2805	0401	-
1485-EBV	171	160	154	222	0407	1501
COX	544	482	735	703	0301	-
PGF	54	150	76	46	1501	-

Antigen presenting cells pulsed with tumor lysate or 100 uM peptide overnight,

then washed prior to assay, all cells at 1x10⁶/ml

FIGURE 5

Determining the P1 and P6 Anchor Positions for Ty 56-70

		GM-CSF (pg/ml/24h.)		
		Exp. 1	Exp. 2	Exp. 3
Ty 56-70	Q N I L L S N A P L G P Q F P	295	524	903
I58	. . Q	<8	<8	<8
	. . F	39	46	ND
	. . V	ND	ND	154
L59	. . . Q	<8	<8	<8
	. . . F	<8	<8	ND
	. . . V	ND	ND	<8
L60	. . . . Q	<8	62	<8
	. . . . F	86	<8	ND
	. . . . V	ND	ND	10
A63	. . . . Q	<8	<8	ND
	. . . . V	889	2080	ND
P64	. . . . Q	<8	<8	ND
	. . . . V	<8	48	ND
L65	. . . . Q	24	50	ND
	. . . . V	2050	3816	ND
Ty 57-70	. . . .	ND	ND	615
Ty 58-70	. . . .	ND	<8	<8
Ty 59-71	. . . . >	ND	<8	<8
Ty 60-72	. . . . >	ND	<8	<8
Ty 61-75	. . . . >	ND	<8	ND
1088mel Lysate		6762	>5500	ND

TIL & EBV backgrounds for Exp.1=53, Exp.2=296, Exp.3=99 pg/ml.
These have been subtracted from the numbers shown above.

FIGURE 6

Ty 56-70

Q N I L L S N A P L G P Q F P

Ty 448-462

D Y S Y L Q D S D P D S F Q D

FIGURE 7

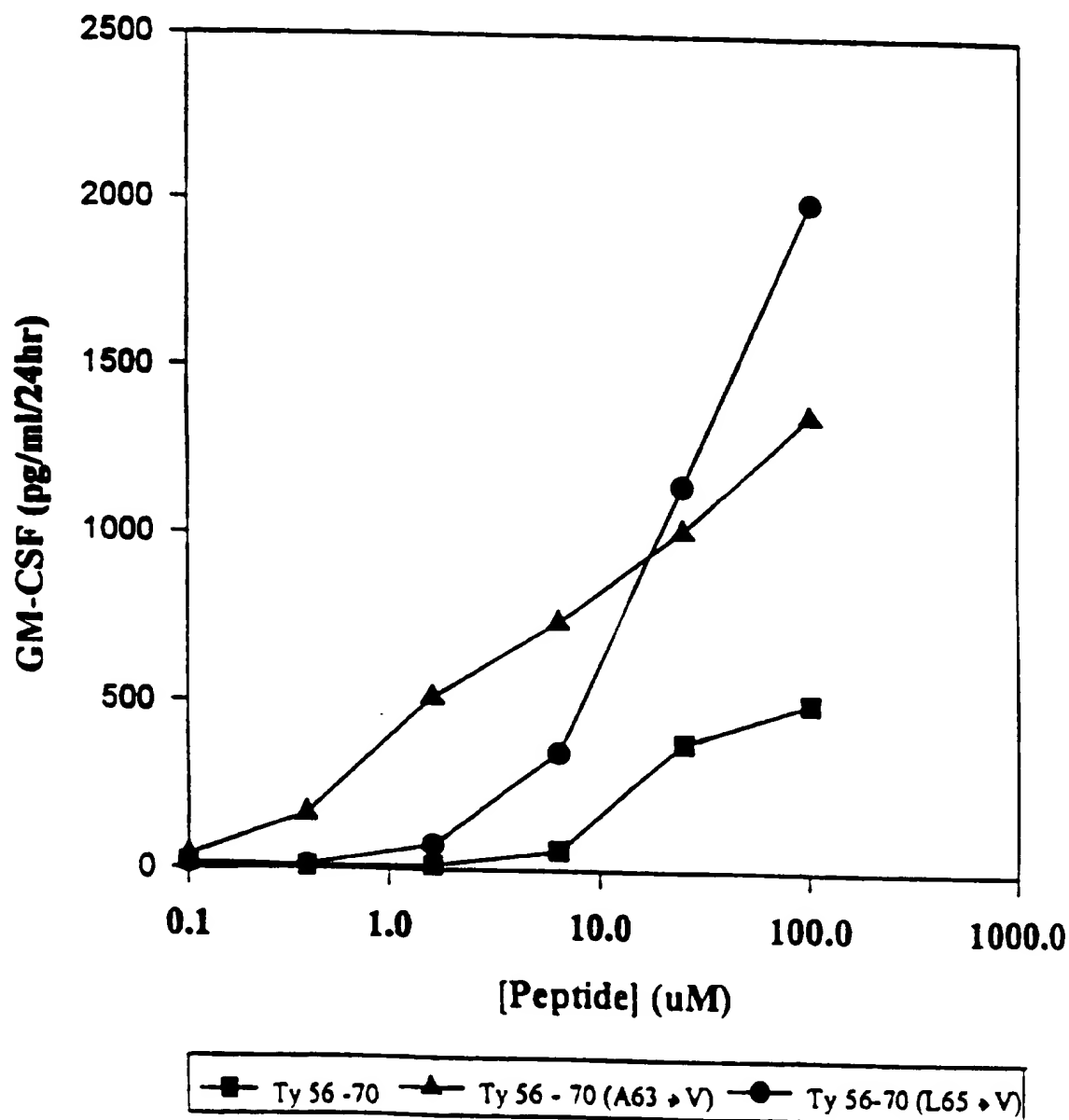## CD4+ 1088 TIL RECOGNIZE MODIFIED
TYROSINASE 56-70 PEPTIDES

FIGURE 8

Determining the P1 Anchor for Ty 448-462

		GM-CSF (pg/ml/24h.)	
		Exp. 1	Exp. 2
Ty 448-462	D Y S Y L Q D S D P D S F Q D	108	802
Y449	- Q - - - - - - - - - - - - -	74	520
	- F - - - - - - - - - - - - -	103	781
Y451	- - - Q - - - - - - - - - - -	28	<8
	- - - F - - - - - - - - - - -	324	1065
L452	- - - - Q - - - - - - - - - -	14	<8
	- - - - F - - - - - - - - - -	14	<8
Ty 449-462	- - - - - - - - - - - - - -	485	1960
Ty 450-462	- - - - - - - - - - - - -	556	2749
Ty 451-462	- - - - - - - - - - - -	21	<8
Ty 452-462	- - - - - - - - - - -	18	<8
1088-mel Lysate		>5500	5822

TIL & EBV backgrounds for Exp.1=52 and Exp.2=196 pg/ml.

These have been subtracted from the numbers shown above.

FIGURE 9

CD4+ TIL 1088 RECOGNIZE MODIFIED
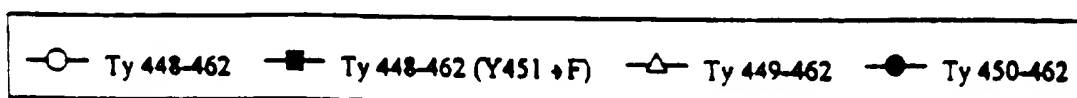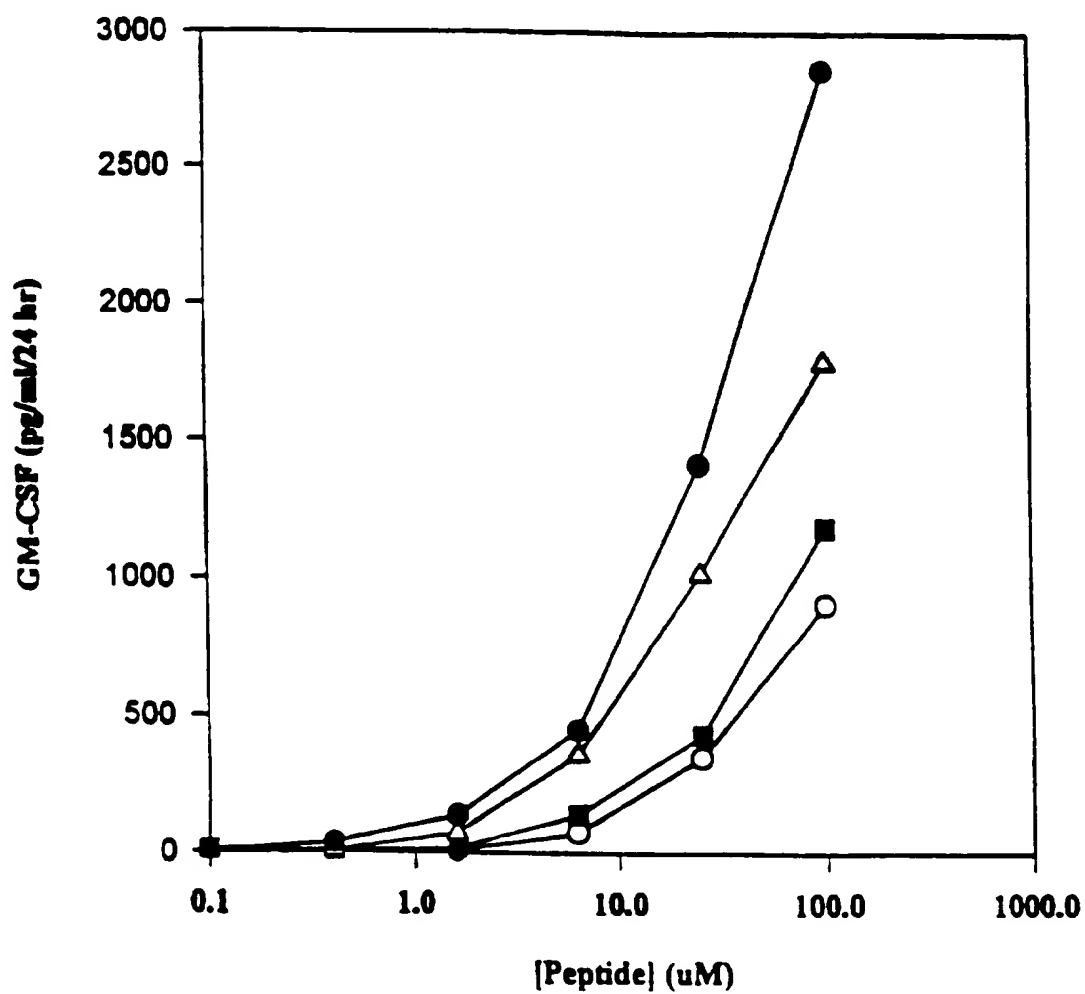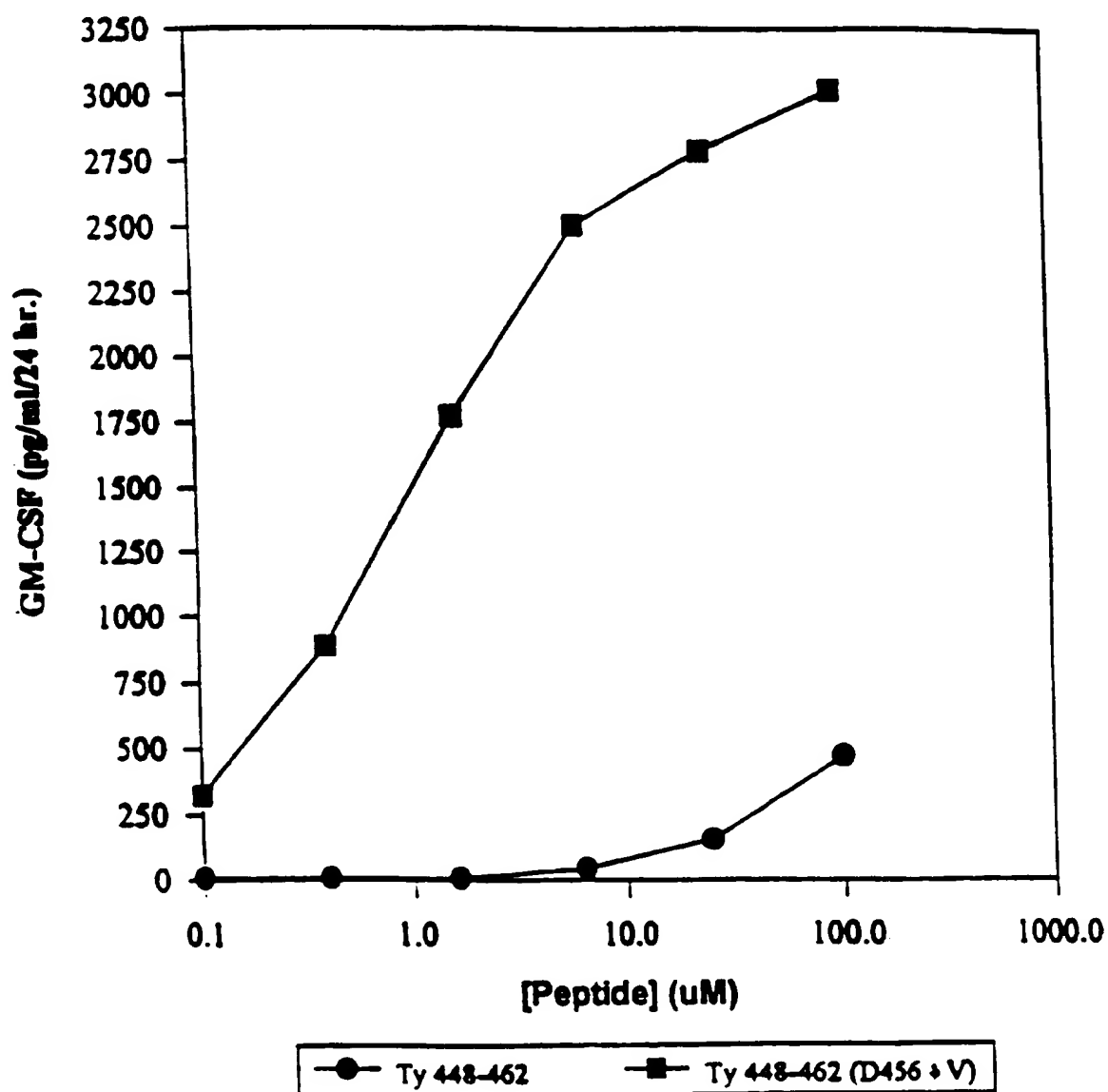## TYROSINASE 448-462 PEPTIDES

FIGURE 10

CD4+ TIL 1088 Recognize Modified Ty 448-462

FIGURE 11

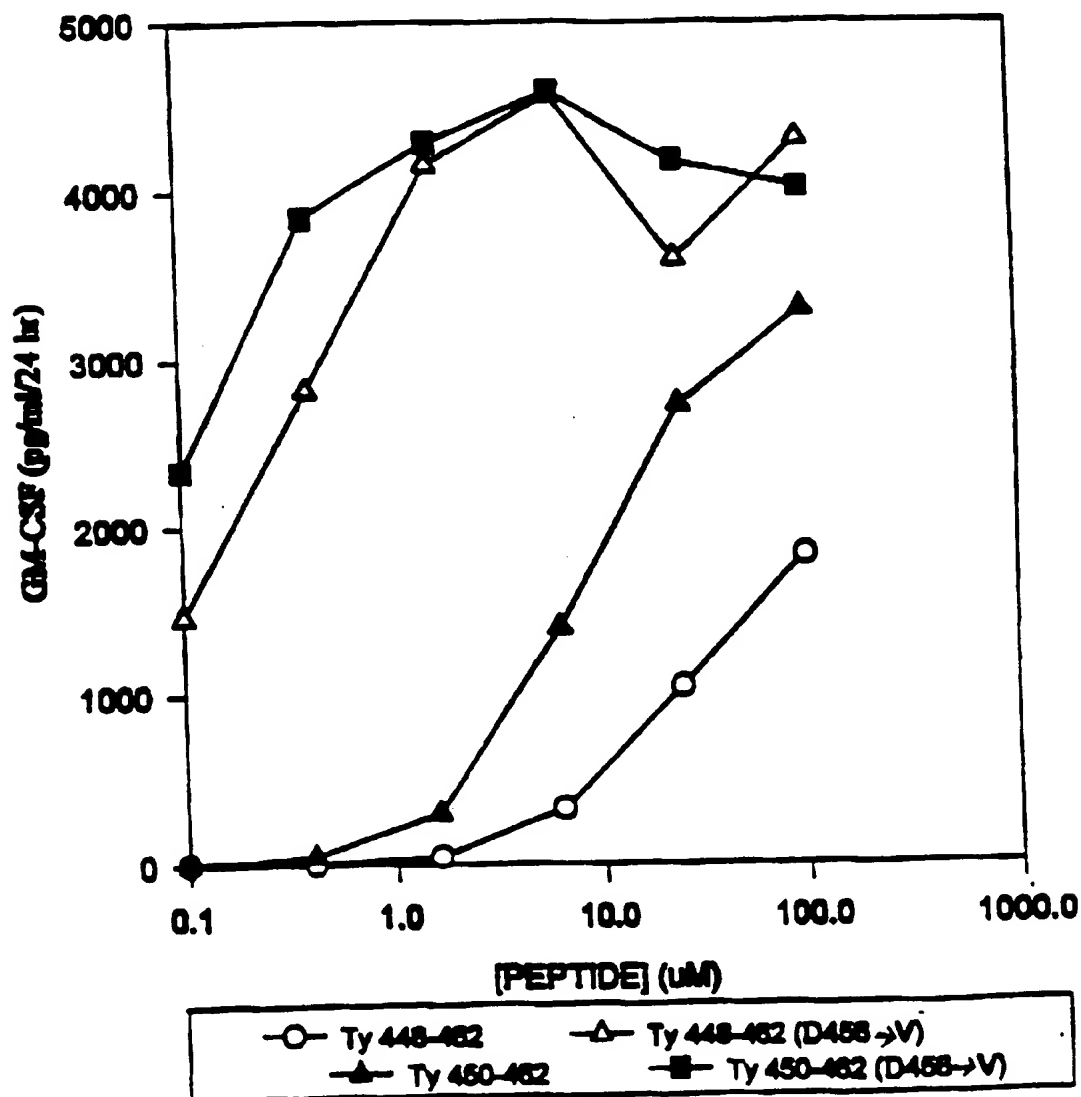## CD4+ TIL 1088 RECOGNIZE MODIFIED
TYROSINASE 448 - 462 PEPTIDES

FIGURE 12